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Whey protein oxidation: LC–MS investigations of peptide markers

Tuuli Koivumäki

ACADEMIC DISSERTATION

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ABSTRACT

Milk whey proteins, especially α-lactalbumin and β-lactoglobulin, are valuable to human nutrition due to their excellent amino acid composition. For this reason, they are also favored by sports nutrition industry. In food products, proteins often constitute with a desired technological function, such as their water-binding, foaming and emulsifying properties. Oxidative deterioration of proteins alters these properties, and can occur during processing and storage. Perhaps even more importantly, oxidation of proteins can lead to their compromised bioavailability and loss of nutritive value, if the so called essential amino acid residues are affected.

In order to evaluate the oxidation status and quality of food proteins, versatile analytical tools are needed. The currently available methods, also used in milk studies, include a very general spectrophotometric measurement of carbonyl compounds which are typically formed by many oxidation reactions. However, the milk whey proteins are particularly rich in amino acids such as cysteine, methionine, tryptophan and tyrosine, which are all known to produce oxidation products other than carbonyl form. More accurate tools are needed to allow a better fit for the different types of proteins, and to expand the understanding of site-specific oxidation reactions.

The purpose of this dissertation study was to establish a novel protocol for the investigation of protein oxidation *via* peptides produced by trypsin digestion. The proteins used in the study were α-lactalbumin and β-lactoglobulin, the two main ingredients of bovine milk whey. The selected peptides were chosen based on their amino acid composition containing at least one residue known to be oxidatively prone. Fractionation was achieved with a developed preparative-HPLC-MS method, and the collected peptides were oxidized individually in metal-catalyzed oxidation (MCO with Fenton chemistry) conditions for 14 days in +37 °C. Furthermore, LC–ESI-QIT-MS^n methods including UV/VIS and fluorescence detectors, were created to investigate the site-specific oxidation modifications in the studied peptides.

The results gathered in this study showed that carbonyl formation was often secondary to other amino acid oxidation modifications in the chosen peptides. Only 50% of the included lysine residues were observed to oxidize into the most typical carbonyl form, whereas all of the studied methionine residues as well as most tryptophan and cysteine residues were found to be very prone to oxidize. In addition to the confirmed main target sites of MCO, this study proposes several promising peptides that could be used as markers of oxidation for monitoring the oxidative status of full whey proteins. Especially the proposed peptide markers formed from ALPMHIR and LIVTQTMK of β-lactoglobulin as well as LDQWLCEK and VGINYWLAHK from α-lactalbumin were considered highly potential. In addition, the created and proven analysis protocol provides a valuable basis for further investigations of food protein and peptide oxidation and interaction studies.

PREFACE

This study was carried out at the Department of Food and Nutrition, formerly known as the Department of Food and Environmental Sciences along with Division of Food Chemistry (until 1.1.2018). The work was mainly funded by the Academy of Finland, which is hereby gratefully acknowledged.

Through my University years in Food Science I have been fortunate to get to participate in several research projects with hands-on analysis of many different food components. I started working as a Research assistant already half-way my M.Sc. studies, analyzing plant steryl esters and steryl ferulates, tocols (vitamin E) and betaine, with varying analytical protocols. My Master's thesis work with folates introduced me to the fascinating world of mass spectrometry, and that bittersweet liaison has lasted ever since. Only the target analytes have been scaled up from small vitamin compounds to peptides, the building blocks of full proteins. While still working on this Ph.D. I got the golden opportunity to jump to the EU-funded project BACCHUS, to do some polyphenolic research and method development, in what I like to call a "pre-doc post-doc" -project. Sometimes it's not all about the destination, but the journey to get there your way… Finally the time came to finalize this dissertation thesis that began in the original project: "Application of LC–MS and HPLC-analytics to study oxidation mechanisms of food proteins", and the result is hereby respectfully presented to the reader.

I owe my deepest gratitude to my supervisor, Professor Marina Heinonen, for allowing me to embark this Ph.D. journey, as well as the phenolic escapade, and for always trusting in my work and way of working. I have especially appreciated that your door was always open to enter if my thoughts ever got tangled, and I would always leave the room with all knots opened. You really have almost a magical talent for putting things in perspective, and I thank you for always keeping up the positive approach in everything you do.

I wish to sincerely thank Professor Vieno Piironen for the additional support and guidance through all my years in food chemistry. In particular, your help with the newly changing administrative jungle has been invaluable, and allowed me to meet all the dead-lines as intended.

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I would like to express my friendly gratitude to Docent Velimatti Ollilainen, Docent Anna-Maija Lampi and Dr. Petri Kylli for the many advice as well as inspiration during the method development and analytical issues of this study. A special thank you is directed to Dr. Marjo Poutanen for introducing me to the new views of protein and peptide research also outside our own Department. In addition, "my" two former M.Sc. students and present Ph.D. student colleagues, Göker Gürbüz and Bei Wang, deserve a big thank-you for your special collaboration. I wish you all the best in finalizing your own dissertation studies! Furthermore, Miikka Olin is thankfully credited for the technical teamwork.

During the years I have had the pleasure to work amongst many wonderful colleagues and friends. Especially I would like to thank Dr. Bhawani Chamlagain, Dr. Minnamari Edelmann, Docent Susanna Kariluoto, Dr. Mari Lehtonen, Docent Ndegwa Maina, Dr. Tanja Nurmi, and Doctoral student Marjo Pulkkinen for your support, guidance, good laughs and some deeper discussions. All highly appreciated! I have also very much enjoyed the company of the staff's weekly floorball games and handicraft get-togethers – you are all too many to mention! Furthermore, it gives me great pleasure to see that the enjoyable working atmosphere we had in D-building is now continuing in the EE-building together with the old and new co-workers. The University really is a unique place to work and it has been a privilege to share this time with all of you.

I would like to extend my gratitude to my friends outside the research community, who kindly keep reminding me for the other important things in life. You have literally kept me going by, for example, calling in the late afternoon: "Hey, researcher, get out of the lab and let's go for a walk in the forest with the dogs!" Thank you for bearing with me and my somewhat colorful mind through the years.

And finally, I could not have done this without my patient parents, Matti and Terja, my always helpful brother Pyry and my grandmother, the iMummu Anita. Thank you for all your support and help through the years, both with my dogs and way beyond…

Helsinki, February 2018

Tuuli Koivumäki

"A scientist in his laboratory is not a mere technician;
he is also a child confronting natural phenomena
that impress him as though they were fairy tales."

- Marie Curie -

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ABBREVIATIONS

α-La	Alpha-lactalbumin
AAS	Alpha-aminoadipic semialdehyde
β-Lg	Beta-lactoglobulin
BPC	Base peak chromatogram
DAD	Diode array detector
diOia	Dioxindolylalanine
diTyr	Dityrosine
DNPH	2,4-Dinitrophenylhydrazine
DOPA	3,4-Dihydroxyphenylalanine
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
FLD	Fluorescence detector
GGS	Gamma-glutamic semialdehyde
HPLC	High-performance liquid chromatography
IAA	(Dietary) indispensable amino acid
LC–MS	Liquid chromatography–mass spectrometry
MALDI	Matrix-assisted laser desorption ionization
MCO	Metal-catalyzed oxidation
MDA	Malondialdehyde
MS^n	Tandem mass spectrometry in time (feature of QIT)
N-fku	*N*-formylkynurenine
Oia	Oxindolylalanine
PDA	Photodiode array (detector)
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PTM	Post-translational modification
QIT	Quadrupole ion trap
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
UV/VIS	Ultra violet/Visible light
ZQ	Single quadrupole (MS)

For a list of all the amino acids and their abbreviations (three and one letter) see Table 1 on page 13.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals **I-III**:

I Koivumäki T, Gürbüz G, Poutanen M, Heinonen M. 2012. A Novel LC–MS Application to Investigate Oxidation of Peptides Isolated from β-Lactoglobulin. Journal of Agricultural and Food Chemistry 60:6799-805.

II Wang B, Koivumäki T, Kylli P, Heinonen M, Poutanen M. 2014. Protein–Phenolic Interaction of Tryptic Digests of β-Lactoglobulin and Cloudberry Ellagitannin. Journal of Agricultural and Food Chemistry 62:5028-37.

III Koivumäki T, Gürbüz G, Heinonen M. 2017. Tryptophan and Cysteine Oxidation Products Dominate in α-Lactalbumin-Derived Peptides Analyzed with LC–MS^n. Journal of Food Science 82:2062-9.

Contribution of the author to papers I to III:

I The author planned the study together with the other authors and performed most of the experimental work. She had the main responsibility of interpreting the results, writing the manuscript and she is the corresponding author of the paper.

II The author planned the study together with the other authors. She participated in the experimental work, acquisition of data and interpretation of the peptide results. She also contributed to the preparation of the manuscript; thus, she was the second author of the paper.

III The author planned the study together with the other authors and performed all of the experimental work. She had the main responsibility of interpreting the results, writing the manuscript, and she is the corresponding author of the paper.

1 INTRODUCTION

Oxidation reactions in foods and food ingredients have been of research interest for a long time. Especially lipids have carried the blame on deterioration of many food products. Current trend, however, has finally turned the importance of understanding protein oxidation reactions "from a poor cousin to a hot topic", as recently worded by Estévez and Luna (2017). Likewise to lipids, protein oxidation occurs mainly during processing and storage, and can be initiated and accelerated by multiple pathways, mostly involving reactive oxygen species (ROS). The main outcomes of unwanted protein oxidation are losses of nutritive value, bioavailability and the desired technological function. On the other hand, purposeful oxidation such as enzymatical modifications can be introduced to improve the structure of foods (Buchert et al. 2010). Generally, protein oxidation products are often less obvious to the consumer than lipid oxidation products, since many of them do not result in compounds directly affecting the sensory quality, such as off-flavors.

Proteins are commonly added to the foods because of their technological properties, such as for emulsifying, water-binding, foaming, gelling and solubility purposes. Especially in low-fat foods, proteins can be added to improve the smooth mouth-feel. The promotion of protein-rich foods has led to the current trend of new innovations of foods with high protein content, which are not only targeted to the conscious consumer. In addition, the high-flying fitness trend is benefited by the sports nutrition industry, with many protein isolate powders and protein-rich food products on the market. Oxidation changes in food proteins can directly affect the shelf-life of all of these products in stores. Furthermore, the potential in bioactive peptides as food ingredients, such as the identified antioxidative, antimicrobial or ACE-inhibitory peptides derived from β-lactoglobulin, has been recognized (Pihlanto-Leppälä 2001; Hartmann and Meisel 2007). However, there is still no health claim accepted in EU on peptides.

According to the testimony of WHO/FAO/UNU (2007) the dietary indispensable amino acids include His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, and in some cases also Cys and Tyr. Oxidation of these amino acid residues in any food protein can significantly reduce the overall nutritional quality. Also the bioavailability of dietary proteins may be compromised due to oxidation, if changes extend to total or partial unfolding of the protein's tertiary structure, or additional intra- or intermolecular cross-linking. This can block the gastric enzymes from fully recognizing their counterparts and result in a failure to execute complete digestion.

In order to be able to assess the oxidative status of dietary proteins, appropriate analytical tools are required. Currently, there is only one generally used routine method in food protein oxidation monitoring. This is based on a spectrophotometric measurement of the protein carbonyls, via derivatization with 2,4-dinitrophenylhydrazine (DNPH; Levine et al. 1990 and 2000). While the formation of protein carbonyls is usually an irreversible process and can successfully represent the oxidation status in some foods, such as certain meat products, in other food types the formation of non-carbonyl compounds may prevail. Unfortunately the DNPH-method also does not differentiate between the origins of the measured carbonyl compounds, and thus the presence of many lipid interaction products and other aldehydes and ketones such as Maillard compounds can lead to overestimation. Furthermore, some studies

suggest that certain amino acids, especially Met, may actually be regulating the oxidation pathways leading to the formation of protein carbonyls (Levine et al. 1999; Moskovitz and Oien 2010; Törnvall 2010). All this leads to the current understanding that one universal analytical method does not exist, and no method should be chosen without knowledge of the target food's protein and amino acid composition.

A more modern approach to protein oxidation analyses involves the use of mass spectrometry (MS). For example, MS has been successfully used in pinpointing the most relevant protein carbonyl products, such as the known semialdehydes of Lys, Pro and Arg (Estévez et al. 2009). However, mastering a complex food sample full of proteins and their various oxidation modifications has turned out challenging. Strong MS data-interpretation skills and expensive equipment may be needed to grasp a large set of data. A more feasible approach is the involvement of protein-specific marker peptides used to represent and indicate changes in the overall oxidation status. Currently, more evidence is needed to improve the understanding of appropriate peptides and oxidation target sites.

The two main milk whey proteins, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), contain several amino acids known to be susceptible for oxidation, as well as potentially result in products that are not of carbonyl form. These include for example Cys, Met, Phe, Tyr and Trp, which are all known target sites in metal-catalyzed oxidation (MCO; Stadtman and Levine 2003). Thus far, photo-oxidation of milk and dairy proteins has been more in the spotlight (Dalsgaard et al. 2007; Scheidegger et al. 2010), yet the presence of transition metals in foods may lead to a preference of different oxidation pathways by MCO. Furthermore, photo-oxidation reactions usually begin from the surface of the protein, progressing along the elevated solvent exposure into the inner regions of the proteins, whereas MCO reactions can be initiated also in the hydrophobic sections, in the presence of bound metal ions such as Cu^{2+} and Fe^{2+}. For the investigation of MCO in potential marker peptides, both α-La and β-Lg can be enzymatically hydrolyzed (digested). Trypsin is a well-known enzyme in protein research, as well as naturally present in the human gastric system. The cleavage of proteins by trypsin is usually very effective and repeatable, resulting in peptides that have Lys and Arg as terminal amino acids on the carboxyl side, unless they were preceded by Pro. In digestion of α-La (14.2 kDa) and β-Lg (18.3 kDa), trypsin produces several peptides less than 2 kDa in size. This is an advantage for mass spectrometric analysis applications.

The purpose of this dissertation study was to establish a novel protocol including LC–MS tools for the investigation of protein oxidation via peptides produced by trypsin digestion. The proteins analyzed in the study were α-La and β-Lg, the two main ingredients of bovine milk whey, and both with desired nutritional value as components in many foods. The results of this study help to understand site-specific MCO modifications in dairy peptides and proteins. To be able to evaluate whether the currently measured carbonyl products properly reflect the oxidation status of α-La and β-Lg, better understanding is needed of the most typical amino acid side chain oxidation reactions in these proteins. In addition, the nominated promising marker peptides could be used as indicators of oxidation of their respective proteins. Furthermore, the created analysis protocol can be utilized in further investigations of protein oxidation and peptide interaction studies.

2 REVIEW OF THE LITERATURE

2.1 Bovine milk whey proteins

The actual protein composition of bovine milk is essential to both the nutritional value and technological properties. The protein composition is mainly determined by genetics, but also varies by season, mainly due to stage of lactation, as well as changes in feed and health (Bobe et al. 1999).

The main proteins found in bovine milk are α_{S1}-Casein (α_{S1}-CN), α_{S2}-Casein (α_{S2}-CN), β-Casein (β-CN), κ-Casein (κ-CN), ɣ-Casein (ɣ-CN), α-La, β-Lg, and serum albumin (SA). In addition, there are small amounts of immunoglobulins (Ig) not so unique to milk, lactoferrin (LF) and a secretory component protein (SC) (Farrell et al. 2004; Horne 2017). Generally 80% of the bovine milk proteins constitute of caseins. The remaining 20% are called 'whey proteins' although this term is a rather general classification, including any milk proteins, whey or serum, remaining soluble at pH 4.6 and 20 °C. According to Horne (2017) this whey fraction traditionally includes β-Lg (~45% of whey), α-La (~20% of whey), SA, Ig and some proteose-peptone fractions, but proteolytic casein fragments may also be found. On the other hand, LF should be included to whey proteins by its amino acid chain homology, although it is not usually present in the soluble fraction (Farrell et al. 2004).

Considering that the dietary indispensable amino acids – also sometimes referred in the literature as the essential amino acids – constitute 47% of the total amino acids in β-Lg (52% if also Cys and Tyr are included) and 47% in α-La (59% with Cys and Tyr), these two main whey proteins are nutritionally an important fraction to the dairy industry. Their tertiary structures can be seen in Figure 1 and comparison of the amino acid compositions in Table 1.

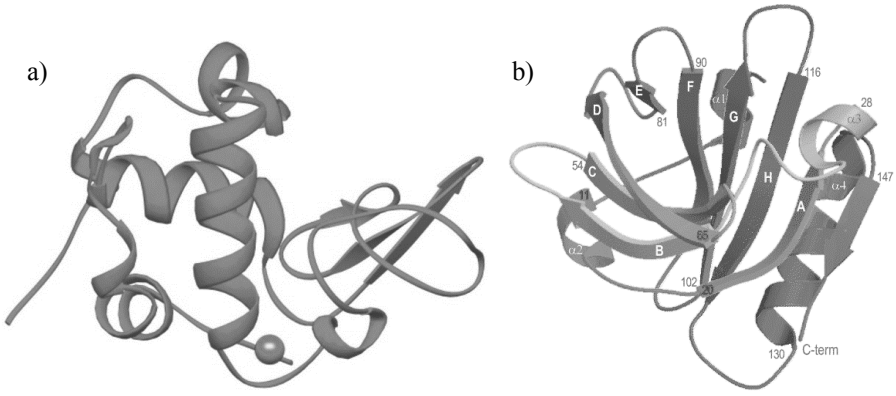


Figure 1. The tertiary structures of a) α-La (including the Ca-binding site) and b) β-Lg. Reprinted from a) Bu et al. 2001, with permission from Elsevier, and b) Oliveira et al. 2001, with permission from John Wiley and Sons.

Table 1. Composition of amino acids in bovine α-La and β-Lg from their most typical variants (A and/or B) in the Scandinavian cattle.

Amino acid			Bovine α-La (B)	%	Bovine β-Lg (A/B)	%
Alanine	Ala	A	3	2.4	14/15	8.6/9.3
Arginine	Arg	R	1	0.8	3	1.9
Asparagine	Asn	N	8	6.5	5	3.1
Aspartic acid	Asp	D	13	10.6	11/10	6.8/6.2
Cysteine(*)	Cys	C	8	6.5	5	3.1
Glutamic acid	Glu	E	7	5.7	16	9.9
Glutamine	Gln	Q	6	4.9	9	5.6
Glycine	Gly	G	6	4.9	3/4	1.9/2.5
Histidine*	His	H	3	2.4	2	1.2
Isoleucine*	Ile	I	8	6.5	10	6.2
Leucine*	Leu	L	13	10.6	22	13.6
Lysine*	Lys	K	12	9.8	15	9.3
Methionine*	Met	M	1	0.8	4	2.5
Phenylalanine*	Phe	F	4	3.3	4	2.5
Proline	Pro	P	2	1.6	8	4.9
Serine	Ser	S	7	5.7	7	4.3
Threonine*	Thr	T	7	5.7	8	4.9
Tryptophan*	Trp	W	4	3.3	2	1.2
Tyrosine(*)	Tyr	Y	4	3.3	4	2.5
Valine*	Val	V	6	4.9	10/9	6.2/5.6
			123	100	**162**	100

* The dietary indispensable amino acids (IAA) in human nutrition (WHO/FAO/UNU, 2007).

2.1.1 α-Lactalbumin

α-La is the second major whey protein in bovine milk, with a compact globular structure and molecular weight of 14 186,06 g/mol (average mass). One α-La protein unit consists of 123 amino acids folded into 9 helices, 4 β-strands and 2 turns. This tertiary structure is further strengthened with intra-molecular disulfide bonds between Cys6-Cys120, Cys28-Cys111, Cys61-Cys80, and Cys73-Cys91, leaving no free thiol groups. The protein has a high affinity binding site for calcium, also stabilizing its secondary structure. This has been assigned to the sequence part DKFLDDDLTDDI (Asp78-Ile89), especially due to the aspartic acid residues Asp82, Asp84, Asp87 and Asp88, as well as Lys79 (Chrysina et al. 2000). In addition, a typical glycosylation site is known at Asn45, allowing the formation of N-linked glycoproteins from α-La.

Three genetic variants of bovine α-La have been identified, of which variant B is clearly dominating and presented as a full reference sequence in Figure 2. The amino acid composition of variant B is used in comparison in Table 1. Variant A is only known in the Australian Droughtmaster cattle breed, and the only difference is Gln10 instead of Arg10 (Bell et al. 1970). Variant C has one Asp substituted to Asn, but it is so rare that the exact placement in

the sequence remains unknown (Farrell et al. 2004). α-La has a theoretical pI of 4,80. The protein typically exists in monomeric form and is not very prone to either acidic or thermal denaturation.

In milk, the main function of α-La is related to lactose synthesis, via enhancing the enzymatic activity of galactosyltransferase. On the other hand, α-La is one of the typical causes of cow's milk allergy in humans. In the literature, several physiological functions have been reported to involve either α-La or its hydrolysates, such as antimicrobial activity (Pellegrini et al. 1999), opioid activity (Teschemacher et al. 1997), reduction of stress (Markus et al. 2000), antihypertensive properties (Pihlanto-Leppälä 2000), activity against gastric mucosal injury (Matsumoto et al. 2001) as well as colon inflammation (Yamaguchi et al. 2014), cell growth regulation (Sternhagen and Allen 2001), and immunomodulation (Cross and Gill 2000).

EQLTKCEVFR ELKDLKGYGG VSLPEWVCTT FHTSGYDTQA IVQNNDSTEY
1 11 21 31 41
GLFQINNKIW CKDDQNPHSS NICNISCDKF LDDDLTDDIM CVKKILDKVG
51 61 71 81 91
INYWLAHKAL CSEKLDQWLC EKL
101 111 121

Figure 2. The sequence of α-La dominant variant B (based on ExPASy UniProtKB, protein accession number P00711, entry name LALBA_BOVIN). Abbreviations of all the amino acids are listed in Table 1.

2.1.2 β-Lactoglobulin

β-Lg is the most abundant component of whey in bovine milk, has a globular structure and molecular weight of 18 281,21 g/mol (average mass). The 162 amino acids of β-Lg produce 7 helices and 14 β-strands, as well as two disulfide bonds existing between Cys66-Cys160 and either Cys106-Cys119 or Cys106-Cys121. Therefore, one free thiol group remains available at Cys119 or Cys121.

Altogether 11 genetic variants have been reported for β-Lg, of which variant B is typically used as the reference sequence and thus presented in Figure 3. Variant C is only found in the Jersey breed and variant W is a "silent" variant due to the only difference of Leu56 instead of Ile56, with no functional change, as reported by Godovac-Zimmermann et al. (1990). Differences between all the known β-Lg variants are summarized in Table 2.

The Scandinavian cattle are known to carry both A and B variants of β-Lg, and generally their milk is considered AB-type. In the study of Iggman et al. (2003), the average β-Lg ratio in milk samples bought from local stores in Finland, Sweden, Norway and Denmark was found to be 42% of variant A and 58% of variant B. However, the ratio is very dependent on the cattle breed. In an earlier study of Lien et al. (1999) the traditional Eastern, Western and Northern

Finncattles were shown to carry protein alleles of 95%, 90% and 81% for variant B, whereas the Finnish Ayrshire and Finnish Holstein-Friesian carried 62% and 52% for variant B, respectively. This is considered consistent with other European cattle breeds, as regular interbreeding across the continent has been done for some decades (Iggman et al. 2003). As a reference to the European cattle, Bobe et al. (1999) studied Holstein-Friesian cows in the USA, reporting that 48% of them were AB-type, 40% were BB-type and 12% AA-type of β-Lg (n = 233 cows).

The only differences in variant A are Asp64 and Val118 instead of variant B having Gly and Ala, respectively. This has led to different thermal aggregation properties between the two variants (Brownlow et al. 1997). The total amino acid compositions of A and B variants are compared in Table 1. The theoretical pI of β-Lg variant B is 4,83, whereas variant A has one more negatively charged Asp residue. The protein molecule prefers its monomeric form only below pH 3.0, and more typically, under physiological conditions, it is found as a homodimer (Sakurai and Goto 2002).

The structural similarity of β-Lg to plasma retinol-binding protein was discovered from horse β-Lg studies by Godovac-Zimmermann et al. (1985) and again presented as a novel finding by Papiz et al. (1986). Indeed the protein seems to be involved in binding and transporting vitamin A and is today classified to the lipocalin family (Farrell et al. 2004). The exact biological function of β-Lg still remains unknown, but is considered to involve binding and transportation of small hydrophobic molecules, such as palmitate (Wu et al. 1999) and 12-bromododecanoate (Qin et al. 1998). This major substrate binding site has been located in the central cavity of β-Lg (Wu et al. 1999), but smaller interaction sites are also known closer to the surface (Brownlow et al. 1997). New evidence suggest β-Lg may also be involved in vitamin D transport *in vivo*, enhancing the effectivity of supplementing milk with vitamin D (Yang et al. 2008 and 2009). Interestingly though, β-Lg is not present in human milk. Alongside to α-La, also β-Lg can cause cow's milk allergy to humans.

```
LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP
1          11         21         31         41
EGDLEILLQK WENGECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK
51         61         71         81         91
KYLLFCMENS AEPEQSLACQ CLVRTPEVDD EALEKFDKAL KALPMHIRLS
101        111        121        131        141
FNPTQLEEQC HI
151        161
```

Figure 3. The sequence of β-Lg dominant variant B (based on ExPASy UniProtKB, protein accession number P02754, entry name LACB_Bovin). In the other dominant β-Lg variant in Scandinavian cattle, variant A, the sequence position 64 has aspartic acid (D) instead of glycine (G), and position 118 has valine (V) instead of alanine (A). Abbreviations of all the amino acids are listed in Table 1.

Table 2. The differences of all 10 known β-Lg protein variant amino acid sequences compared to the universal reference sequence of variant B.

β-Lg	Differences in amino acid sequence (Variant B in parenthesis)		
Variant A	Asp64 (Gly)		Val118 (Ala)
Variant C	His59 (Gln)		
Variant D	Gln45 (Glu)		
Variant E		Gly158 (Glu)	
Variant F	Ser50 (Pro)	Gly158 (Glu)	Tyr129/130 (Asp)
Variant G	Met78 (Ile)	Gly158 (Glu)	
Variant H	Asp64 (Gly)	Asn70 (Lys)	Val118 (Ala)
Variant I	Gly108 (Glu)		
Variant J	Leu126 (Pro)		
Variant W	Leu56 (Ile)		

2.2 Protein oxidation pathways in foods

Protein oxidation in foods occurs mainly during processing and storage. Several factors are known to trigger, and sustain, protein oxidation reactions, of which many fall under the description of reactive oxygen species (ROS), such as hydroxyl radical ($HO^{\bullet}$), hydroperoxyl radical ($HOO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), superoxide radical anion ($O_2^{\bullet-}$), and the non-radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and ozone (O_3). For decades, protein oxidation was considered "secondary" to lipid oxidation, mainly due to the less obvious end products that do not always result in off-flavors or color changes in the food products. Nowadays it is understood that protein oxidation often goes hand in hand with lipid oxidation, for example through same ROS, as well as alkyl radicals ($RC^{\bullet}HR$), alkylperoxy radicals ($RCOO^{\bullet}$) and alkyl peroxides (RCOOH) of either lipid or protein origin. The oxidation rates are greatly dependent on the total composition of the food, and especially the amount of lipids versus proteins. Even co-oxidation is known to occur between lipids and proteins, such as transferring of radicals and thus the oxidation potential (Schaich 2008). Additionally, the resulting oxidation product can be a combination of both, such as the typical malondialdehyde (MDA) induced Schiff base adducts of β-Lg peptides studied by Gürbüz and Heinonen (2015).

Metal-catalyzed oxidation

Regarding food proteins, peptides and amino acids, MCO is considerably the most researched approach in oxidation studies. Free transition metals, mainly iron and copper, are available in food systems to bind with the proteins, and in the presence of oxygen or hydrogen peroxide, can produce ROS such as hydroxyl radicals $OH^{\bullet}$. Perhaps the most renowned MCO mechanism is the Fenton chemistry; the reaction between iron and hydrogen peroxide, first noted more

than 100 years ago. The exact mechanism theory still remains unproven and controversial (Barbusinski 2009), but the classical Fenton reaction is typically explained according to equation 1 (IUPAC Recommendations 1997). Additionally, the reaction is recognized via an oxoiron(IV) intermediate, presented in equation 2:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O \quad (2)$$

The resulting ferryl species $[Fe(IV)O]^{2+}$, or sometimes known as $[Fe(OH)_2]^{2+}$, from reaction 2 is an active oxidant as well, and can react further by for example two-electron oxidation of alcohols to ketones (Pestovsky and Bakac 2004; Prousek 2007). The ferryl ion is also capable of reacting with hydrogen peroxide, in order to reduce back to Fe(II) (equation 3). For the Fe(III) ions and complexes from the classical Fenton reaction (1), the reduction back to Fe(II) can occur with superoxide, known as the Haber–Weiss reaction (or Superoxide driven Fenton reaction), and explained in equation 4. Also ascorbate is an excellent reducing agent, thus often added to the MCO research study setups to enhance the reaction cycle. In presence of ascorbate, the ferric ion readily reduces back to ferrous ion *via* the formation of an ascorbate radical (equation 5). Both reactions 4 and 5 are also important metal-redox cycling mechanisms in biology (Du et al. 2012).

$$FeO^{2+} + H_2O_2 \rightarrow Fe^{2+} + H_2O + O_2 \quad (3)$$

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \quad (4)$$

$$Fe^{3+} + AscH^- \rightarrow Fe^{2+} + Asc^{\bullet -} \quad (5)$$

The reactions are also considered a rather "caged" cycle, where the ROS generated prefers to react with functional groups nearby the original metal-binding site in the protein (Chevion 1988; Stadtman 1990). According to the current knowledge, some kind of equilibrium between the presented reactions 1 and 2 most likely prevails in reality, depending on other available conditions such as pH and disposal of further reagent components (Barbusinski 2009). Therefore, the description of 'Fenton or Fenton-like chemistry' is used to explain the MCO reactions so often observed. The amino acid residues considered to be most prone to MCO modifications are Arg, Cys, His, Lys, Met, Pro, and Tyr (Stadtman 1990). In addition, Phe and Trp are highly susceptible targets of the ROS generated by MCO reactions (Stadtman and Levine 2003). Also according to Stadtman (1990) peptide bond cleavage is not a typical consequence in proteins exposed to MCO.

Photo-oxidation and radiation induced oxidation

The numerous pathways of photo-oxidation in proteins have recently been extensively described by Pattison et al. (2012). In brief, the direct oxidation reactions can be caused by UVA (λ 315-400 nm) but more often UVB (λ 280-325 nm) radiation, and basically affect only a few absorbing amino acid residue targets (Trp and Tyr), typically leading to electron transfer and hydrogen abstraction mechanisms. In addition, photo-induced singlet oxygen ($^1O_2$) can initiate further oxidative reactions, for example radical, peroxide and carbonyl formation. The

main targets of photo-induced ROS formed are His, Cys, Met and Phe, with actual direct UV damage being less significant (Pattison et al. 2012). Once commenced, the processes often continue even after a short exposure to radiation.

The third radiation category, UVC (λ 100-280 nm), is used for non-thermal sterilization purposes to reduce the microbial load in foods, especially in liquids. Scheidegger et al. (2010) studied the effect of UVC to whole and skim milk proteins, and found the target amino acids to be consistent with those reported from UVB. Changes were detected in all levels, primary to tertiary structures, with emphasis on protein aggregation due to diTyr formation, fragmentation due to peptide bond cleavage and Trp oxidation to various products including formation of the harmful *N*-formylkynurenine (*N*-fku).

Many foods also contain other ingredients with intrinsic chromophores, for example porphyrins (such as heme) and flavins (containing pteridine), which may either enhance or quench the ROS reactions. Especially in milk and other dairy products the presence of sensitizers such as riboflavin, protoporphyrin IX and certain chlorophyll derivatives are known to significantly enhance the formation of ROS (Aurand et al. 1966; Dalsgaard et al. 2007; Pattison et al. 2012; Wold et al. 2015). Interestingly though, Jung et al. (2000) reported that addition of 0.1% ascorbic acid to aqueous β-Lg solution – instead of enhancing the ROS formation – significantly prevented the oxidation of Cys, His, Ser, Gly, Asp, Arg, Pro, Phe and Trp, but not Lys, Ile, Glu, Val and Met, in a riboflavin-sensitized setup. All in all, photo-oxidation seems to be the favored approach for many research groups studying milk and dairy proteins (Dalsgaard et al. 2011; Scheidegger et al. 2016; Dyer et al. 2017).

In addition to UV-radiation, also the effect of fluorescent light exposure to milk has been studied, for example by Scheidegger et al. (2010). Furthermore, direct radiation with X-rays and γ-rays are also known to produce hydroxyl radicals in food systems (Stadtman and Berlett 1997). The oxygen radicals produced by high energy radiation can lead to random fragmentation in several structural levels of the proteins and peptides.

Enzymatic oxidation

Enzymatic oxidation of proteins can be considered from different angles; via the H_2O_2 formed by endogenous oxidases, as a site-specific modification tool in for example cross-link formation, or via protein-to-protein radical chemistry. For instance Østdal et al. (2000) studied the combined effect of endogenous lactoperoxidase (LPO) in unpasteurized milk, with and without added H_2O_2. The amount of protein radicals formed was observed to depend on the addition of H_2O_2, and more dityrosine was detected to form in β-Lg than casein. However, protein radical formation was detected even in control solution of LPO and H_2O_2, possibly indicating that this enzyme contributed to the overall oxidation through general radical chemistry rather than any actual direct enzymatic interaction reactions. Purposeful modification of food proteins via enzymatic oxidation is a very contemporary research interest in the food industry (Buchert et al. 2010).

Reactive nitrogen species induced oxidation

Similarly to ROS, also free radicals formed by reactive nitrogen species (RNS) can contribute to oxidation in foods, under suitable surrounding conditions. Among the typical RNS are nitrogen monoxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals, as well as peroxynitrite anion ($ONOO^-$) and its protonated conjugate peroxynitrous acid (ONOOH). Especially peroxynitrite is an active oxidant, and is formed from the reaction of $^{\bullet}NO$ and superoxide ($O_2^{\bullet-}$). In food proteins, the main targets of peroxynitrite include Cys, Met and Tyr (Alvarez et al. 1996; Alvarez and Radi 2003).

2.2.1 Modifications of protein conformation

Oxidation of proteins by ROS can give rise to further ongoing reactions that ultimately lead to some degree of denaturation of the protein. The loosening of the tertiary structure can reveal amino acid side chains highly susceptible for oxidation by themselves, or expose a likely counterpart for inter- or intra-protein cross-linking, such as Tyr or Cys (Partanen et al. 2011). Even direct interaction of two carbon-centered protein or peptide radicals can form a new cross-linked structure (Garrison 1987). Most of the covalent cross-links formed are not easily broken, which may lead to the protein not being able to fold back to the native state anymore (Stadtman and Levine 2003).

Several studies suggest that digestive enzymes including trypsin and chymotrypsin are more eager to degrade oxidized proteins, because of their resulted (partial) denaturation and thus elevated area exposed to solvents, compared to the unoxidized equals (Davies et al. 1987; Stadtman 1990). At the same time, heavy aggregation or complex formation can lead to loss of solubility. Also gastrointestinal digestion of oxidized proteins may be significantly delayed, if oxidation has modified the protein conformation in a way that enzymes are hindered. This is especially true if protein aggregation via inter-Tyr-Tyr or -S-S- cross linking has been formed and the tertiary structure has been unfold as a consequence of oxidation (Stadtman 1990).

2.2.2 Cleavage of peptide bonds

Free radical-mediated oxidation of proteins and peptides can lead to cleavage of peptide bonds. The underlying mechanisms have been thoroughly presented by Garrison (1987) and later reviewed by Stadtman and Levine (2003). The protein alkoxyl radicals and alkylperoxides initiated by ROS can continue to peptide bond scission by two general reaction trails: the α-amidation or diamide pathways. Cleavage by the α-amidation route generally results in amide and α-keto-acyl fragments, whereas cleavage by the diamide pathway forms diamide and isocyanate derivatives. In addition to these, a few side chain oxidation reactions of hydroxyl radicals can eventually lead to peptide bond cleavage; namely the oxidation of glutamyl and aspartyl residues presented by Garrison (1987). According to Uchida et al. (1990), also the oxidation of proline to prolyl residues may continue to further fragmentation of the protein *via* the peptide bonds.

In MCO systems, however, the oxidation reactions are more likely oriented towards the local amino acid side chains close to the metal binding sites in the proteins, and thus very little peptide bond cleavage is typically observed, as underlined by Stadtman (1990). Therefore it is important to distinguish that peptide bond cleavage by oxidation reactions is significantly more typical for proteins rich in proline, glutamic and aspartic acids, that are processed under conditions in which any alkoxyl and alkylperoxide derivatives (originating from lipids or proteins) are likely to be forming.

2.2.3 Amino acid side chain modifications due to oxidation

Site-specific oxidation of the amino acid side chains is the essence in understanding protein oxidation. All of the 20 common amino acids, the building blocks of all food proteins, are susceptible to oxidation by ROS, but generally some are considered more prone than the others. For example the aliphatic hydrophobic amino acid side chains of Ala, Ile, Leu, Val and the exceptionally short Gly are usually not the primary targets. In MCO systems, the amino acids commonly classified as most prone to oxidize are the sulfur-containing Cys and Met, the aromatic Tyr, Trp, Phe and His, as well as Pro, Lys and Arg (Stadtman 1990; Stadtman and Levine 2003). Interestingly, most of them (especially His, Lys, Met, Phe, Trp) are also classified as dietary indispensable to human nutrition (WHO/FAO/UNU 2007), which means that any oxidation modifications directly affect the nutritional quality of such a protein or food item.

Carbonyl derivatives are among the conventional oxidation products of many amino acid residues, such as Arg, Lys, Pro and Thr. However, these amino acids do not represent the majority in all foodstuffs such as in milk, thus broader understanding of oxidation reactions are needed. Some characteristic oxidation modifications of amino acid side chains typical in α-La and β-Lg are summarized below in this chapter. The main modifications reflected in the results and discussion of this dissertation (studies **I-III**) are collected in Table 3 (see also chapter 2.3.4 and Table 4).

Table 3. Some of the characteristic side chain oxidation modifications of amino acids typical in α-La and β-Lg, and essential to this dissertation study, listed in alphabetical order. Specific MS fragmentation sites are given for His, Met, Pro and Trp (*N*-fku).

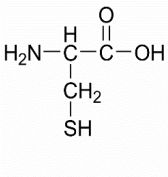
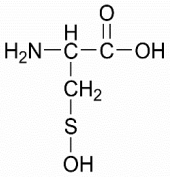
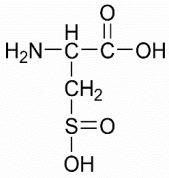
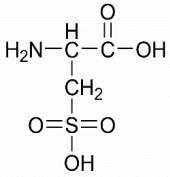
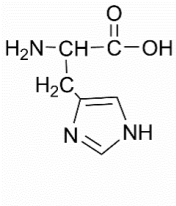
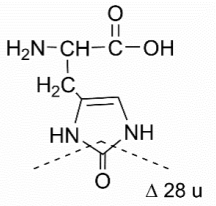
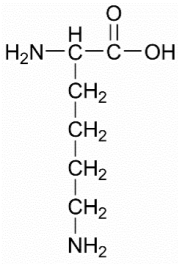
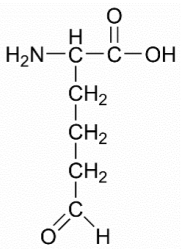
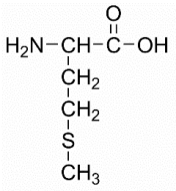
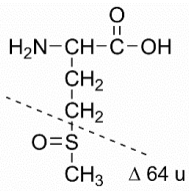
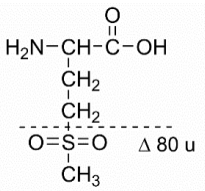
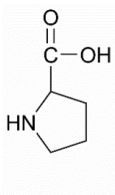
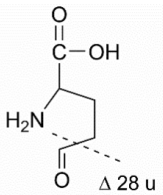
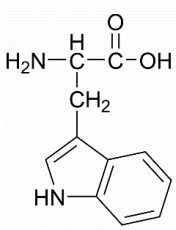
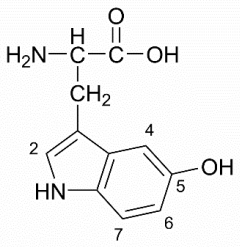
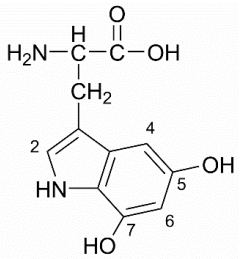
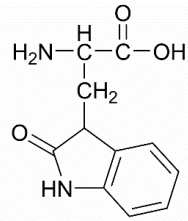
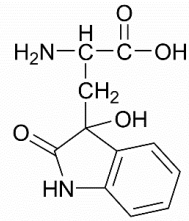
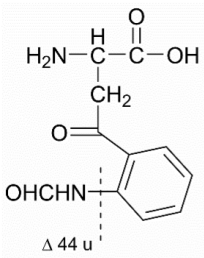
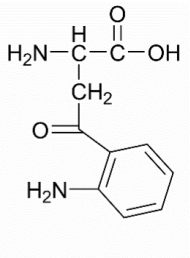
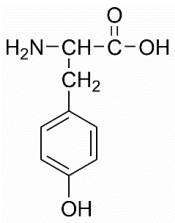
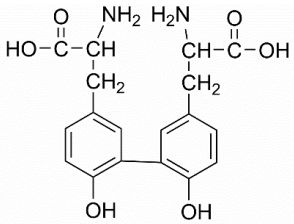
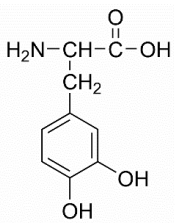
Amino acid	Side chain oxidation modifications		
			
Cysteine 121 u	Cysteine sulfenic acid 137 u (Δ +16 u)	Cysteine sulfinic acid 153 u (Δ +32 u)	Cysteine sulfonic acid (Cysteic acid) 169 u (Δ +48 u)
			
Histidine 155 u	2-oxo-histidine 171 u (Δ +16 u)		
			
Lysine 146 u	α-aminoadipic semialdehyde (AAS) 145 u (Δ -1 u)		
			
Methionine 149 u	Methionine sulfoxide 165 u (Δ +16 u)	Methionine sulfone 181 u (Δ +32 u)	
			
Proline 115 u	γ-glutamic semialdehyde (GGS) 131 u (Δ +16 u)		

Table 3. (continued)

Amino acid	Side chain oxidation modifications	
		
Tryptophan 204 u	5-Hydroxytryptophan (2-/4-/6-/7-OH-Trp) 220 u (Δ +16 u)	5,7-Dihydroxytryptophan (2-/4-/5-/6-/7-diOH-Trp) 236 u (Δ +32 u)
		
...from Trp	Oxindolylalanine (Oia) 220 u (Δ +16 u)	Dioxindolylalanine (diOia) 236 u (Δ +32 u)
		
...from Trp	*N*-formylkynurenine (*N*-fku) 236 u (Δ +32 u)	Kynurenine 208 u (Δ +4 u)
		
Tyrosine 181 u	Dityrosine (diTyr) 360 u (Δ +179 u)	3,4-Dihydroxyphenylalanine (DOPA) 197 u (Δ +16 u)

Tyrosine

The phenolic side chain of Tyr is a typical target of ROS (Stadtman and Levine 2003), although Giulivi et al. (2003) name the aromatic amino acids less likely to be affected by MCO than by radicals from ɣ-radiolysis. The main products of Tyr side chain oxidation in MCO conditions are 3,4-dihydroxyphenylalanine (DOPA), dityrosine (diTyr) and trityrosine cross-linking (Giulivi et al. 2003), but also formation of 2,3-dihydroxyphenylalanine has been reported by Maskos et al. (1992) from their Fenton chemistry experiments. Other typical modifications of Tyr include chlorotyrosine and 3-nitrotyrosine from oxidation reactions such as RNS (Stadtman and Berlett 1997).

DiTyr and cross-linking of Tyr to other potential amino acid residues can also be formed enzymatically. This may even be a desired modification for the food product, such as improving the texture of the product (Buchert et al. 2010). For example tyrosinase can oxidize Tyr (and DOPA) into a quinone, which is then able to form cross-linking with other Tyr, Lys and Cys residues, both intra- and inter-protein (Selinheimo et al. 2008; Partanen et al. 2011). Interestingly, both α-La and β-Lg seem to be rather resistant to enzymatic cross-linking, most likely due to their compact structure (Gauche et al. 2008). The possibility to cross-link another dairy protein, β-casein, was studied by Monogioudi et al. (2009), with reflection to thickening of milk products or use as food hydrocolloids.

Tyr cross-link formation to diTyr (homo-Tyr-Tyr) is one of the only established oxidation markers available and in use by the biomedical industry for oxidative stress induced changes (Wells-Knecht et al. 1993; Giulivi and Davies 2001). Specific detection methods can be developed due to the strong fluorescent properties of diTyr. In the food industry, the use of diTyr as a marker of oxidation has yet been quite modest. However, studies such as Østdal et al. (2000) have successfully followed diTyr formation in unpasteurized milk where H_2O_2 was added with purpose.

Phenylalanine

Oxidation of the aromatic Phe by the Fenton chemistry, as well as ɣ-radiolysis, has been shown to result in mainly 2- and 3-hydroxyphenylalanine as well as 4-hydroxyphenylalanine – also known as tyrosine (Maskos et al. 1992; Stadtman and Levine 2003). Maskos et al. (1992) also found the oxidation of Phe to be readily influenced by pH, with maximum reactivity observed at pH 5.5. A direct "lack of hydroxylated products" was reported around pH-level 8, meaning that oxidation of Phe in foods may be heavily influenced by the actual food product in question. *In vivo* studies have confirmed that the conversion of Phe to Tyr-derivatives is possible also enzymatically (Udenfriend and Cooper 1952). Their use as markers of oxidative status may therefore be problematic.

Cysteine and methionine

Cys and Met have in common a special feature in their side chains: the sulfur-atom (S). From mass spectrometric interest, S is known to have isotopic diversity, namely, there are four stable isotopes known (with natural abundances in parentheses): ^{32}S (94.93%), ^{33}S (0.76%), ^{34}S (4.29%) and ^{36}S (0.02%). Further prospects of S are the several oxidation states (-II, II, IV, VI) with its maximum valence of 6. This gives S several possibilities to oxidize also in the protein side chains of Cys and Met. In Met, however, the S exists between two methyl groups leaving only 4 valence electrons available, but this site is known to be one of the most vulnerable sites to ROS of many origins (Stadtman and Levine 2000), including MCO, as well as peroxynitrite and alkyl/lipid hydroperoxides (Davies et al. 1999). The primary oxidation product is Met sulfoxide, which can continue further to the dioxidized form, the Met sulfone (Table 3). The formation of these two oxidation products may be of safety concern for example in UV-treated milk (EFSA Scientific opinion 2016), especially due to the irreversible nature of Met sulfoxide, which can only be reduced enzymatically (Moskovitz et al. 1996). In the study of Cilliers et al. (2014) of bovine milk treated with heat and UV-light, Met sulfone was actually found a much significant marker of oxidation than Met sulfoxide. This may be special to dairy products or foods in general, as in biological systems Met sulfoxide has typically been the product of interest (Davies et al. 1999). The increasing polarity of Met sulfoxide and sulfone, compared to unoxidized Met, has been considered to result in opening of the previously buried hydrophobic pockets, thus exposing the protein for further oxidation reactions. This connection of Met oxidation possibly regulating the formation of protein carbonyls has been extensively reviewed by Moskovitz and Oien (2010).

In Cys, the sulfur is located at the end of the side chain, thus allowing the possibility to oxidize in three stages forming Cys sulfenic, sulfinic and sulfonic acids (also known as Cysteic acid). At least Cys sulfonic acid has been shown to be a rather stable end product, whereas Cys sulfenic and sulfinic acids are generally quite reactive (Claiborne et al. 1999). In addition, Cys is rather eager to form disulfide bonds both intra- and inter-protein. These covalent bonds forming the cross-linking are essential to the tertiary structure and thus also the processing properties of many food proteins.

The high oxidation tendencies of Cys and especially Met are considered to be connected to self-regulation of many biological proteins. Met oxidation to Met sulfoxide is reversible and this cyclic reaction, in biological systems catalyzed by available reductases, is also consuming available ROS thus protecting the rest of the protein (Moskovitz et al. 1998; Stadtman 2006). Similar redox regulation functions have been proposed for Cys in cellular processes (Claiborne et al. 1999). Little is yet known if related regulation applies to foods, but many antioxidant properties of food peptides are indeed connected to Met as one of the main target amino acid side chains, such as in the study of both α-La and β-Lg derived antioxidant peptides by Hernández-Ledesma et al. (2005).

Additionally, both Cys and Met oxidation have been connected to the formation of off-flavors in milk. Photo-oxidation of proteins especially in the presence of riboflavin can result in

Strecker degradation products to form methional, dimethyl disulfide and various sulfur and aldehyde compounds that are problematic for the sensory quality of the milk product (Dalsgaard et al. 2007).

Histidine

The side chain of His is highly sensitive to MCO conditions, and the main pathway leads to 2-oxo-His (Amici et al. 1989; Uchida and Kawakishi 1993). In the interesting study of Schöneich (2000), His oxidation as part of a protein/peptide sequence is discussed from many angles. Evidence suggests that $HO^{\bullet}$ generated in presence of transition metals produces several oxidation products such as Asp, Asn and formylasparagine, but the inclusion of ascorbic acid directed the oxidation towards 2-oxo-His formation, even though ascorbic acid was not required for its production. It may thus be that in order for 2-oxo-His to be formed, the position of His in the protein sequence must be favorable. Schöneich (2000) even pointed out that if the His in question is itself metal-bound, it does not need to be located on the surface of the protein to undergo MCO. In biological applications, the use of 2-oxo-His as a biomarker has been suggested (Uchida and Kawakishi 1993).

Lysine, Arginine and Proline

Formation of carbonyl compounds is a typical consequence of protein oxidation (Stadtman and Levine 2003). Among the main target residues in MCO are Lys, Arg and Pro, all of which have in common a similar formation pathway to carbonyl-containing semialdehydes as their major end-products (Amici et al. 1989). According to the review of Stadtman and Levine (2003), the semialdehyde from Lys is considered to form through the interaction of Fe(II) directly with the amino group of Lys. The available H_2O_2 can produce a $OH^{\bullet}$ that readily attacks this binding site, generating the α-aminoadipic semialdehyde (AAS) via deamination. Similarly, the γ-glutamic semialdehyde (GGS) can be formed from the side chains of Arg and Pro (Amici et al. 1989).

Other MCO products from Pro include the 4- and 5-hydroxyprolines, although their formation was found marginal compared to the GGS conversion (Amici et al. 1989). According to the Unimod database (Creasy and Cottrell 2004), 3-hydroxyproline, 4-hydroxyproline and 3,4-dihydroxyproline are the confirmed post-translational modifications of Pro, but resulting from enzymatic prolyl hydroxylase activity, or acid hydrolysis (Amici et al. 1989), and thus not considered further in this study. In addition, the formation of a 2-pyrrolidone has been reported in the literature (Uchida et al. 1990).

The AAS and GGS have been shown to comprise majority of the total carbonyl compounds from e.g. rat liver proteins (Requena et al. 2001) and some meat and dairy proteins, including α-La (Estévez et al. 2009).

Aspartic acid and glutamic acid

Unlike Arg, His and Lys, all of which carry a positive charge in their side chains, the negatively charged amino acids Asp and Glu are not generally considered very prone to ROS. Literature is scarce on their own oxidation reactions, especially of food origin, but Drakenberg et al. (1983) reported to have been the first to identify the formation of 3-hydroxyaspartic acid (Hya) as a PTM to Asp in a bovine protein C. Technically, a similar oxidation reaction could happen in the side chain of Glu as well. However, the importance of Asp and Glu to MCO systems may actually arise from their ability to bind metal ions, thus offering a potent oxidation site affecting the amino acids nearby. As metal binders in proteins, Glu and Asp can actually act as monodentate, bidentate, or bridging ligands depending on the available metal ion (Bertini and Turano 2007). Naturally, a completely bound metal ion is less susceptible to ROS.

Tryptophan

The aromatic amino acid Trp is a highly prone target for many oxidation modifications, also in peptides and proteins. However, $OH^{\bullet}$ radicals generated by radiolysis seem to cause a different relative rate of oxidation than MCO, and also the favored end products can vary according to prevailing conditions (Stadtman and Berlett 1991). The main oxidation products of Trp are usually named in the literature as the 2-, 4-, 5-, 6-, and 7-hydroxyl derivatives, as well as kynurenine and *N*-fku (Stadtman and Levine 2003). Thus, both the phenyl moiety and the pyrrole ring of Trp are considered as the most susceptible sites for oxidation, but depending on oxidation conditions (Simat and Steinhart 1998). Other typical oxidation modifications include the formation of oxindolylalanine (Oia), dioxindolylalanine (diOia), and 3a-hydroxypyrroloindole-2-carboxylic acid (PIC). MS based characterization of oxidation products typical in Fenton chemistry conditions confirmed the main monohydroxy-Trp, dihydroxy-Trp and *N*-fku modifications, but also revealed the possibility of dimer formation (Domingues et al. 2003). Both, the Trp-Trp dimer similar to the diTyr as well as Trp-Trp-OH dimerization were observed.

Oxidation modifications of Trp, both free and protein bound, have been widely studied through the years (e.g. Krogull and Fennema 1987; Finley et al. 1998; Simat and Steinhart 1998; Domingues et al. 2003; Salminen 2009; Todorovski et al. 2011). The challenges usually evolve around the available analytical tools, and in attempts to distinguish between the many different oxidation products (Friedman and Cuq 1988). For example in MS based methods the trial is that all of the five monohydroxyl derivatives result in a same mass addition of +16 u, as does also the formation of Oia. Furthermore, an observed mass addition of +32 u can belong to either diOia, *N*-fku or any combination of the dihydroxyl derivatives. In some studies, chromatographic separation of the similar compounds has been achieved, and characteristic fragment ions have been found to help with the differentiation (Todorovski et al. 2011). As the aromatic Trp itself possesses both characteristic UV absorbance and fluorescent properties, the changes observed can be benefited from in identification studies (Salminen et al. 2008). For example, the loss of Trp fluorescence properties as an indicator of Trp destruction in UVC-irradiated whole and skim milk was studied by Scheidegger et al. (2010).

Oxidation of Trp in foods is of special interest due to the adverse health effects connected especially to *N*-fku, such as promotion of the onset of urinary bladder carcinogenesis. In addition to *N*-fku, kynurenine and other possible carcinogens from Trp have been studied, found to form and discussed further (Matsukura et al. 1981; Krogull and Fennema 1987; Friedman and Cuq 1988; Stadtman and Berlett 1997). On a recent note, the formation of *N*-fku has been observed in milk products (Scheidegger et al. 2010). On the other hand, promising antioxidant activity has been connected to Trp-containing peptides derived from both α-La and β-Lg (Elias et al. 2005; Hernández-Ledesma et al. 2005). Furthermore, Trp is also one of the amino acids classified as indispensable to human nutrition. Its losses by oxidation reactions therefore greatly affect the nutritional value of any food product.

The remaining amino acids

The amino acids not considered in depth above include Ala, Gly, Ser, Leu, Ile, Val, Thr, Asn and Gln. Although some generic oxidation products from them are recognized in the literature, these amino acids are not considered among the most prone to oxidize in MCO and Fenton chemistry systems. The reported oxidation modifications include for example Leu oxidation to either 3-, 4- or 5-hydroxyleucine (Stadtman 1993), Val to 4-hydroxyvaline, Asn to 3-hydroxyasparagine and Thr to a carbonyl-containing 2-amino-3-ketobutyric acid (Stadtman and Levine 2003).

2.3 Methods for analysis of food protein and peptide oxidation

2.3.1 Spectrophotometric methods

Currently, the only routinely used method for analysis of protein oxidation is the quantification of generic protein carbonyls via their reaction with DNPH. This results in yellow protein-hydrazone derivatives that can be quantified by spectrophotometry at λ 370 nm. The method was originally introduced by Levine et al. (1990), but many improvements have since been suggested. For example Levine et al. (2000) presented ways to add specificity and sensitivity by employing HPLC, gel filtration or Western blotting. Even ELISA can now be employed through the development of DNPH-sensitive antibodies (Buss et al. 1997). The differences in available techniques have been extensively reviewed by Hawkins et al. (2009), however, Estévez et al. (2008) have noted that even the simple procedure is rather time-consuming and not very cost-efficient with the amount of chemicals needed. Thus, the newer simplified procedure introduced by Mesquita et al. (2014) may have reduced this workload by shifting the measurements to a more specific λ 450 nm.

The analysis of protein carbonyls based on the DNPH-method is still widely used to explain oxidation modifications, including milk, dairy products, and milk protein isolates (Fenaille et al. 2006; Semagoto et al. 2014; Scheidegger et al. 2016). However, the measurement of protein carbonyls as marker of oxidation in foods is problematic in many ways. Carbonyls (aldehydes

and ketones) as oxidation products are formed mainly from three amino acid residues; Lys, Arg and Pro. In addition, carbonyl content increases also when cleavage of the peptide backbone of the proteins in the α-amidation pathway occurs (Stadtman and Levine 2000) as well as by many secondary interaction products with lipid oxidation compounds. The DNPH-derivatization step does not differentiate specifically between protein carbonyls, and might lead to an overestimation by compounds of non-protein and of non-oxidation origin. The method may give indication of ongoing oxidative modifications, but knowledge of the total sample composition is needed in order to make any further decisions of the overall status of the food product. In addition, recent mass spectrometric studies such as Milkovska-Stamenova et al. (2017) have indicated that carbonyl formation by MCO is rather scarce and thus cannot be used to explain the effect of thermal processing in different milk types.

2.3.2 High-performance liquid chromatography

The HPLC instrumentation has been a tool of choice for protein oxidation researchers for a long time. Buffer solutions as eluents can be fitted to the chemical properties of proteins and peptides, through pH-value adjusted according to the solubility of the studied proteins. In addition to the protein or peptide fingerprinting analyses by HPLC, fractions can be collected for more detailed analysis.

In HPLC applications, proteins and peptides are generally detected by DAD/UV at λ 210-215 nm, which matches the absorbance of the peptide bonds. Certain amino acids have significant absorbance on higher wavelengths, which can be used for more specific detection and quantification. For example in acidic conditions (pH 3.1) Tyr can be detected by UV at λ_{max} 234 and 274 nm, while one of its main oxidation products, the diTyr at λ_{max} 236 and 284 nm (Giulivi et al. 2003). The exact wavelength to be used in detection is, however, often dependent on pH, and absorption may be hindered by the other amino acid residues (i.e. rest of the protein) located around the targeted residue. Additionally, fluorescence (FL) detection can improve analysis of proteins and peptides containing specific amino acids. DiTyr can be detected precisely from λ_{ex} 315 nm (alkaline solutions) or λ_{ex} 284 nm (acidic solutions) and λ_{em} 415-420 nm (Giulivi and Davies 1994). Tyr oxidation products diTyr and 3,4-dihydroxyphenylalanine have been identified from milk proteins by the successful combination of HPLC-DAD/FLD (Dalsgaard et al. 2011).

Estévez et al. (2008) presented FL as a novel and specific tool to the analysis of both loss of Trp fluorescence and formation of new fluorescent protein oxidation products in muscle protein-containing emulsions. Although this method did not include separation by HPLC, the fluorescence application could be transformed to an in-line detector. Generally in protein analyses, the Trp residues are determined with excitation at λ 280 nm and emission at λ 330-350 nm, such as in the β-Lg + antioxidant study of Salminen et al. (2010). On the amino acid oxidation level, the earlier study of Salminen et al. (2008) presented a combination technique of HPLC coupled with DAD and FL detection for the analysis of Trp and its many oxidation products. The FL was employed with varying settings, according to the individual oxidation product compounds. They could for example clearly show the loss of FL properties by

oxidation of Trp to most of the studied modifications, while still remaining detectable at UV 260 nm.

Besides Trp, most other FL-based techniques require derivatization with a specific reagent, such as AAS and GGS treated with *p*-aminobenzoic acid, for the detection of the protein oxidation carbonyl products of Lys, Arg and Pro with HPLC-FLD (Akagawa et al. 2006). Other applications include for example Lys derivatization with fluorescamine and Arg with 9,10-phenanthrenequinone, but usually no connected to HPLC-FL detection (Hawkins et al. 2009).

The introduction of mass spectrometric tools was first a challenge to protein research, due to certain limitations in choice of eluents. Most buffer solutions are not compatible with the ionization sources, as the non-volatile components soon contaminate the ionization chamber. Furthermore, the development of the atmospheric pressure ionization (API) techniques fully allowed protein and peptide research to be performed via "soft" ionization. Of these API-techniques electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the most used in protein mass spectrometric studies (Törnvall 2010).

The investigation of protein oxidation by Schey and Finley (2000) was among the early side of evolution in the possibilities of LC–MS/MS. They could successfully identify oxidized residues in e.g. bovine α-crystallin, by fragmentation of the oxidized peptides. Another oxidation target analysis by LC–ESI-TOF-MS/MS was used to identify the His residues involved in MCO of a β-amyloid peptide, confirming for example the His formation preference to 2-oxo-His over any Tyr residue modifications (Schöneich and Williams 2002). These type of approaches have since been benefited in trying to understand the many pathways and preferences of protein oxidation. However, even with the powerful MS-tools available today, the combination of different detection techniques coupled to HPLC is often preferred. Estévez et al. (2009) studied AAS and GGS as potential oxidation markers in meat, soy and dairy proteins with their proposed LC-FLD/MS method, and in comparison to the DNPH-method. Among their studied proteins was α-La, where both AAS and GGS were shown to significantly form within the 2 weeks of metal-catalyzed oxidation. Similarly, but without any dairy proteins, LC–MS together with FL detection has been employed as an enhancement tool for identifying and quantifying peptides and proteins containing Phe, Tyr and Trp (Saraswat et al. 2012). All in all it could be summarized, that the HPLC-DAD/FLD/MS -techniques available today have many applications and approaches to offer for the investigation of the different aspects in protein oxidation.

2.3.3 Quadrupole ion trap mass spectrometry in peptide and protein research

The choice of a mass analyzer affects the level of information that can be gained from the analysis. A single quadrupole (ZQ) type of analyzers can be used to simply detect the compound ions based on their mass-to-charge (*m/z*) -ratios. A triple quadrupole (QqQ) mass analyzer is an excellent tool especially for the detection of known modifications, as the available techniques include the possibility to connect a product ion to its mother ion, with the multiple reaction monitoring (MRM) practice. Some mass analyzers, such as the time-of-flight (TOF and QTOF) instruments, offer higher mass resolution, and are thus often used for the

peptide mapping and identification purposes. For example the bovine and porcine β-Lg sequences were compared by the TOF approach of Invernizzi et al. (2006). Also many combination techniques exist, in order to further improve the accuracy and/or allowing tandem fragmentation analyses.

Quadrupole ion trap (QIT) is a unique mass analyzer best fitting for structural analysis (March 2009). Its electrodes form a hyperbolic ring structure with additional two side electrodes, as if a napkin ring had a plate on both sides. The analyte ions are directed from the API source, towards high vacuum, and trapped into the space between the ring structure. By adjusting the voltage and radio frequency (RF) potentials of the electrodes, the ions can be kept in a complex motion trapped inside the potential well for a long time (March 2009). Detection is achieved by scans of ramping the RF amplitude, so that the ions exit the trap in the order of their *m/z*-ratios. When used in-line with an HPLC, the multiple ion scans can be combined into a full scan ion chromatogram. The QIT-MS technique usually has good sensitivity and excellent selectivity.

The advantage of QIT mass analyzers to structural analysis is the possibility to trap a chosen ion inside, perform collision-induced dissociation (CID), and again trap only a certain desired product ion. If the signal is high enough, the product ion can be kept trapped, and fragmented again, several times. This is also known as tandem mass spectrometry in time, or MS^n (Murray et al. 2013, IUPAC Recommendations). For example in peptide analysis, a sequence may be confirmed by this method, as the parameters leading to isolation and fragmentation are well adjustable and correctly set can confirm the relation of a mother ion to a fragment ion, amino acid by amino acid. However, fragmentation by the peptide backbone is not always the typical result of QIT analysis, as also observed by Qin and Chait (1997).

Interestingly, Qin and Chait (1999) later also showed that fragmentation of peptides in QIT analysis had certain patterns depending on the amino acid composition and size. For example peptides containing Pro preferred to fragment on the N-terminal side of Pro. Similarly, peptides having Lys as the C-terminal residue, such as many peptides hydrolyzed with trypsin, showed preference to fragment by cleavage of the terminal Lys, but this was found influenced by the presence of Arg. Such patterns may be benefited from in identification studies. Originally, the QIT instruments were targeted towards higher molecular weight proteins, peptides and other biomolecules, especially due to the excellent combination with the “soft” ionization techniques such as ESI. For quantification purposes, the use of a triple quadrupole instrument may be preferred over QIT, as discussed in the diTyr-biomarker comparison study by Orhan et al. (2005). In dairy protein and peptide research the LC–QIT-MS approach has been used by for example Hernández-Ledesma et al. (2004a,b; 2005) in the identification study of biologically active peptides in fermented milk.

2.3.4 Mass spectrometric tools for data interpretation

Generally, the most used mass spectrometric approach in protein research is the peptide mass fingerprinting procedure. The protein of interest is enzymatically hydrolyzed into peptides smaller than 3 kDa, followed by LC separation of the produced digests for peptide sequencing by either MALDI- or ESI-MS/MS. The fingerprints are then compared to available databases, either intra-laboratory or those accessible via internet, such as the Swiss-Prot from the ExPASy proteomics database. Similar approach can also be used to identify changes in the expected (native) peptide fingerprint, including oxidation modifications. This was the chosen approach of Bridgewater and Vachet (2005) who could thus locate their oxidized residues into three specific peptides. The advantage was that only the pinpointed peptides needed to be chosen for a more detailed fragmentation analysis. Modifications of lactose induced Maillard product formation as well as oxidation in the whey proteins have also been studied with the help of Swiss-Prot/TrEMBL database searches (Meltretter et al. 2007). In this MALDI-TOF-MS approach the incubated proteins were digested with either trypsin or endoproteinase AspN prior to the MS analysis. Furthermore, Cheison et al. (2011) studied the differences in tryptic digestion patterns of β-Lg, under different acidic and thermal conditions. The diverse peptide fingerprints were also analyzed with a MALDI-TOF-MS/MS instrument, and several differences to the database default were seen.

At present, a few protein modification databases are available, which also include a collection of the currently known oxidation modifications to the amino acid residues. These include for example the Unimod protein modification database for mass spectrometry (Creasy and Cottrell 2004), the FindMod tool of ExPASy the bioinformatics resource portal provided by the SIB Swiss Institute of Bioinformatics (Artimo et al. 2012), and Delta Mass, the user contributed database of protein post translational modifications by the Association of Biomolecular Resource Facilities. Unfortunately the use of these databases has some limitations, as many of the modifications known in the literature are also left unrecognized by the searches. The mostly suggested modifications are the oxidation or dioxidation of Met, Cys or Trp. Therefore, interpretation of mass spectra requires additional knowledge from the researcher even if software tools are used for screening of the data. A list of typical oxidation modifications in comparison to those included in the three common databases is presented in Table 4.

The available mass spectrometry data interpretation tools were successfully used for example in the study of Guedes et al. (2009) where MCO incubated BSA residues were investigated with MALDI-TOF/TOF and the Mascot software (Matrix Science) was connected to the Unimod database. The accurate analytical tools allowed not only the identification of final oxidation products, but also the oxidation time-dependent investigation of multiple side chain oxidation products. Thus, they could conclude for example that the increase in carbonyl group formation was not linear, and oxidation seemed to favor the side chains situated close to Cys disulfide bridges, especially Met, Trp, Lys, Arg, Tyr and Pro. Even though BSA contains a good number of amino acids likely oxidizing into carbonyl groups, the understanding of non-carbonyl oxidation products was found equally important, as unfolding of the protein due to oxidation continued. Later on Guedes et al. (2010) used a similar study to investigate MCO modifications in glycated insulin. This time their LC–MS approach could identify mainly

carbonylated products from Pro, His, Val, Leu and Gly. The recent MS related publications on dairy proteins include for example the investigation of whey protein oxidation *via* the Mascot software based data interpretation tools using a program called ProteinScape (Dyer et al. 2017). This method confirmed mainly Cys, Met and Gln oxidation sites in the studied β-Lg and α-La.

Characteristic fragmentation patterns of oxidized peptides have also been studied for the aid of identification. For example Lagerwerf et al. (1996) presented that the fragmentation loss of Δ 64 u is characteristic for peptides containing Met sulfoxide, thus corresponding to the elimination of -CH_3SOH side chain. This fragmentation pattern was also recently used in identification of Met sulfoxide formation in milk whey proteins (Wüst and Pischetsrieder 2016). Similarly, the loss of Δ 80 u was shown characteristic for methionine sulfone-containing peptides, with the elimination of -CH_3SOOH side chain (Lagerwerf et al. 1996). In the case of *N*-fku, the loss of -NHCHO (Δ 44 u) is known as the characteristic fragmentation piece (Domingues et al. 2003). Hopefully, the current mass spectrometric databases could be advanced with the addition of this type of typical fragmentation information of further oxidative modifications. This could significantly improve the data handling and identification of changes in for example the fingerprints. In order to do so, more data is needed.

Quantification with MS tools is not always a straightforward issue. Many factors affect the accuracy in MS based quantification, including ion suppression and matrix effect. The use of an internal standard is not commonly met, but has been successfully used for milk proteins (Wüst and Pischetsrieder 2016). Quantification of the whey proteins β-Lg and α-La has earlier, however, been studied *via* program-operated deconvolution of mass spectra of their native or e.g. lactosylated forms (Czerwenka et al. 2006). Unfortunately, this strategy has proven to be highly reliant on the signal-to-noise -ratio of the spectra of interest, and those protein forms with low concentrations could not be successfully detected by the deconvolution scheme. In order to replace this technique, Losito et al. (2007) have presented a new approach with quantification of the proteins (native or slightly modified) based on multiple ion current extraction. This method was also applied to the comparison of native and lactosylated forms of the whey proteins in pasteurized and UHT milk (Losito et al. 2007). Quantification of lactosylation in β-Lg and α-La was also the interest of Le et al (2013) in their development of a MRM method for a triple quadrupole instrument. Although this method was successful in selectivity, sensitivity and the achieved level of quantification, it required pre-information on the specific modifications to add to the list of targeted reactions. This list became complex when multiple charged precursor ions needed to be included as well. Considering the many oxidation modifications and their combinations available to a single (whey) protein, more information on the preferred target sites is needed if LC–MS based methods are to be implemented.

Table 4. List of common oxidation modifications of the amino acid side chains, and their influence on shift of mass. Inclusion (+) or exclusion (-) of the named modifications are indicated for the three most used databases in software based MS interpretation tools, the Unimod, FindMod (ExPASy) and Delta Mass.

	Δ *m* (u)	Unimod[a]	FindMod[b]	Delta Mass[c]	Reference
Arginine					
γ-Glutamic semialdehyde	-43	+	-	+	(Amici et al. 1989; Berlett and Stadtman 1997; Guedes et al. 2009)
”Oxidation to glutamic acid”	-27	-	-	+	
4-Hydroxyarginine	16		+	-	
”Oxidation/Hydroxylation”	16	+		-	(Guedes et al. 2009)
3,4-Dihydroxyarginine	32		+	-	(Taylor et al. 2000)
”Dioxidation”	32	+		-	(Guedes et al. 2009)
Asparagine					
3-Hydroxyasparagine	16	+	+	-	(Stadtman and Levine 2003; Guedes et al. 2009)
Aspartic acid					
3-Hydroxyaspartic acid	16	+	+	+	(Guedes et al. 2009)
Cysteine					
Oxoalanine	-18	+		-	(Guedes et al. 2009)
3-Oxoalanine	-18		+	-	
Sulfenic acid	16	+	+	+	(Guedes et al. 2009)
Sulfinic acid	32	+	+	-	(Guedes et al. 2009)
Sulfonic acid (Cysteic acid)	48	+	-	+	(Berlett and Stadtman 1997; Guedes et al. 2009)
Glutamic acid					
Hydroxyglutamic acid	16	+	-	-	
Histidine					
Asparagine	-23	+	-	-	(Berlett and Stadtman 1997; Guedes et al. 2009)
Aspartic acid	-22	+	-	-	(Berlett and Stadtman 1997; Guedes et al. 2009)
2-Oxo-histidine	16	+	-	+	(Uchida and Kawakishi 1993; Berlett and Stadtman 1997; Guedes et al. 2009)
Leucine					
3-/4-/5-Hydroxyleucine	16		-	-	(Stadtman 1993)
”Oxidation/Hydroxylation”	16	+	-	-	
Lysine					
α-Aminoadipic semialdehyde (Allysine)	-1	+	+	+	(Berlett and Stadtman 1997; Guedes et al. 2009)
α-Aminoadipic acid	15	+	-	+	(Guedes et al. 2009)
5-Hydroxylysine	16		+		
”Oxidation/Hydroxylation”	16	+		+	(Guedes et al. 2009)
4,5-Dihydroxylysine	32		+	-	(Taylor et al. 2000)
”Dioxidation”	32	+		-	(Guedes et al. 2009)

Table 4. (continued)

	Δ *m* (u)	Unimod[a]	FindMod[b]	Delta Mass[c]	Reference
Methionine					
Methionine sulfoxide	16	+	+	+	(Lagerwerf et al. 1996; Berlett and Stadtman 1997; Guedes et al. 2009)
Methionine sulfone	32	+	+	+	(Lagerwerf et al. 1996; Berlett and Stadtman 1997; Guedes et al. 2009)
Phenylalanine					
"Oxidation/Hydroxylation"	16	+	-	-	(Guedes et al. 2009)
2-/3-/4-Hydroxyphenylalanine	16		-	-	(Berlett and Stadtman 1997)
2,3-Dihydroxyphenylalanine	32	+	-	-	(Berlett and Stadtman 1997; Guedes et al. 2009)
Proline					
2-Pyrrolidone	-28	+	-	-	(Berlett and Stadtman 1997; Guedes et al. 2009)
Pyrrolidinone	-30	+	-	-	(Berlett and Stadtman 1997; Guedes et al. 2009)
Pyroglutamic acid	14	+	-	-	(Berlett and Stadtman 1997; Guedes et al. 2009)
γ-Glutamic semialdehyde	16	+		+	(Berlett and Stadtman 1997; Guedes et al. 2009)
Hydroxyproline	16	-	+	+	
3-Hydroxyproline	16	-	+	+	
4-/5-Hydroxyproline	16	-			(Berlett and Stadtman 1997)
Dihydroxyproline	32	+			(Guedes et al. 2009)
3,4-Dihydroxyproline	32		+	+	
Glutamic acid	32	-	-	+	
Tryptophan					
Kynurenin	4	+	-	+	(Berlett and Stadtman 1997; Guedes et al. 2009)
Oxolactone	14	+	-	-	(Guedes et al. 2009)
2-/4-/5-/6-/7-Hydroxytryptophan	16				(Berlett and Stadtman 1997; Domingues et al. 2003)
3-Hydroxytryptophan	16		+		
"Oxidized tryptophan"	16	+	+	+	(Guedes et al. 2009)
3-Hydroxykynurenin	20	+	-	-	(Berlett and Stadtman 1997; Guedes et al. 2009)
N-formylkynurenin	32	+	-	-	(Berlett and Stadtman 1997; Domingues et al. 2003)
"Double oxidation"	32	-	-	+	
Nitrotryptophan	45	+	-	-	(Alvarez et al. 1996; Berlett and Stadtman 1997)
Tyrosine					
2-Aminotyrosine	15	+	-	-	
3,4-Dihydroxyphenylalanine	16	+	+	+	(Berlett and Stadtman 1997)
Quinone	30	+	-	-	(Guedes et al. 2009)
Trihydroxyphenylalanine	32	+	-	+	
Nitrotyrosine	45	+	-	-	(Berlett and Stadtman 1997)

Table 4. (continued)

	Δ *m* (u)	Unimod[a]	FindMod[b]	Delta Mass[c]	Reference
Valine					
4-Hydroxyvaline	16	+	+	-	(Stadtman and Levine 2003)

[a] Unimod, the public domain database of protein modifications for mass spectrometry http://www.unimod.org

[b] FindMod tool is provided by ExPASy, the Bioinformatics Resource Portal of the Swiss-Prot society. https://web.expasy.org/findmod/

[c] Delta Mass is the database of protein post translational modifications by the Association of Biomolecular Resource Facilities (ABRF). https://abrf.org/delta-mass

2.3.5 Other methods

While the spectrophotometric and HPLC based analytical methods may be the primary tools currently used in protein and peptide oxidation studies, gathering of additional information may be needed. For example the detection of cross-linking formed by oxidation may require a different approach. Cross-linking often leads to changes in secondary and tertiary structures of the proteins, and bigger aggregates can be formed between cross-linking of residues from different proteins. The main amino acids involved in cross-linking modifications are Cys, Tyr, Lys (Stadtman and Levine 2003) and Trp (Domingues et al. 2003). SDS-PAGE or native PAGE can be utilized in separation of these conformational differences, as well as size exclusion chromatography (Törnvall 2010). Also the evaluation of oxidized thiols can be achieved by 1D or 2D gel separation of the affected proteins (Eaton 2006). However, in milk whey proteins this is not the prime concern, as there are no free thiol groups available in native α-La, and only one free thiol group exists in β-Lg (Cys119 or Cys121).

Currently available applications for the detection of particular protein oxidation products include also the ELISA methods (Hawkins et al. 2009). Specific antibodies have been developed especially for the Tyr modifications, both free and protein bound (Kato et al. 2000). These applications are mostly targeted for monitoring of oxidative stress levels in ageing and disease, but considering the strengths and limitations of the techniques, similar assays could be used for monitoring of simple food matrices where same oxidation markers (such as diTyr formation) can be shown to be characteristic (Onorato et al. 1998).

The proteomic approach is another advantage of the gel electrophoresis (1D or 2D), where the single protein spots can be further introduced to LC–MS/MS for characterization and fingerprinting. This also allows the further investigation of oxidation modifications in the altered protein profiles, as recently demonstrated in cooked pork products (Di Luccia et al. 2015). Similar approaches extended to other food types are likely the future of food protein oxidation research.

3 AIMS OF THE STUDY

The overall aim of this dissertation study was to develop novel tools for food protein oxidation research, specifically, a protocol for the in-liquid digestion, chromatographic separation, fractionation, oxidation, and LC–MS analysis of whey proteins. The established analytical approach was employed to investigate metal-catalyzed oxidation (Fenton chemistry) modifications in peptides isolated from α-lactalbumin and β-lactoglobulin. Several of the oxidized peptide products were reflected as potential markers of protein oxidation.

The individual objectives were as follows:

- To develop novel tools for the analysis of protein oxidation from fractionated peptides of chosen whey proteins (**I, II**)
- To investigate and locate MCO target amino acid residues in the studied peptides (**I, II, III**)
- To propose promising protein oxidation marker peptides from α-lactalbumin and β-lactoglobulin (**I, III**)

4 MATERIALS AND METHODS

This section summarizes the materials and methods used and developed in this study. The created overall protocol is presented in Figure 4 and explained in more detail below as well as in the original publications **I-III**.

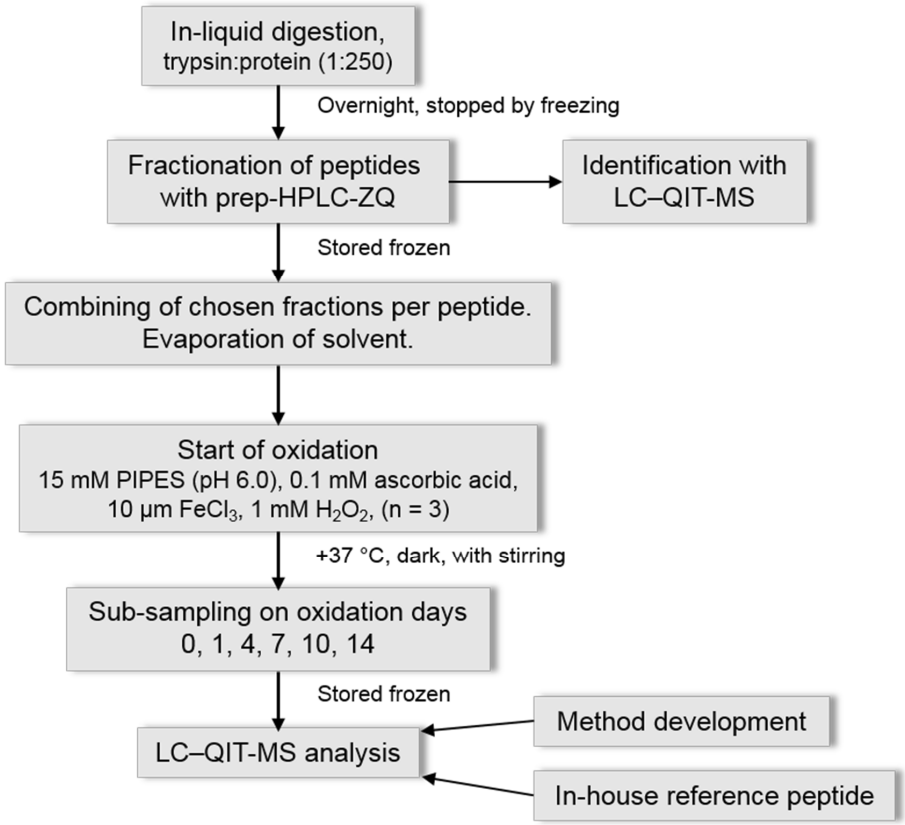


Figure 4. The outline of the developed protocol.

4.1 Reagents and chemicals

The chromatographically purified and lyophilized proteins used in all studies were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Bovine β-Lg contained both of the most typical variants A and B in a ratio of 1:1, and bovine α-La was solely the dominant variant B. Sequencing grade modified trypsin, without significant autolytic activity, was acquired from Promega Corp./BioFellows (Madison, WI, USA). The other chemicals and reagents used in the studies are presented in more detail in the original publications **I-III**.

4.2 Enzymatic hydrolysis (digestion)

In order to produce enough peptides of the desired medium size, an in-liquid digestion protocol was developed in Eppendorf scale (study **I**). 10 mg of protein was first dissolved into 1 mL of 50 mM ammonium bicarbonate (NH_4HCO_3) buffer, pH 8. Lyophilized trypsin was dissolved in its own resuspension buffer (50 mM acetic acid) by shaking, not stirring. The freshly prepared 1 μg/μL enzyme solution was added to the protein sample in the protease:protein ratio of 1:250 (w/w), followed by careful vortexing.

Trypsin is known to cleave specifically from the carboxyl side of Lys and Arg, except when preceded by Pro. Since both β-Lg and α-La contain approximately 10% Lys + Arg, it was found accordingly that trypsin effectively produced several peptides of desired size, by length of the amino acid chain. In pre-testing (data not shown), both the experimented chymotrypsin and pepsin were found less favorable in terms of reproducibility, effectivity, and peptide size. In addition, trypsin is a typical natural enzyme in the human gastric system, and maximally active in the pH range of 7-9. It can be reversibly inactivated at pH below 4 or freezing. The modified trypsin used in the studies was pre-treated against autolysis of the enzyme itself.

The prepared digestion samples were incubated in the dark at +37 °C. During the experimentation, it was found that β-Lg was more robust in required incubation time than α-La. For β-Lg the accuracy of "overnight incubation", approximately 16-20 h, produced relatively repeatable peptide fingerprint chromatograms where the peptides were mostly in the favorable 400-2000 u range. Typically, incubation of ~18 h was used for β-Lg (studies **I-II**). Longer digestion time, 20-22 h, was required for α-La to produce enough of the desired peptides, with closer to 22 h preferred (used in study **III**). Also it was noted that more repeatable peptide profiles were produced for α-La with accurate timing, compared to the more robust β-Lg digestion.

In all cases, the digestion was ceased by freezing the Eppendorf tubes in -20 °C for at least another night before preparative-HPLC analysis.

4.3 Peptide fractionation with preparative-HPLC

The peptides produced in the tryptic in-liquid digestion were chromatographically separated by a developed gradient elution (0.1% formic acid in both water, A, and acetonitrile, B) protocol with fraction collection, presented in study **I**, and with slight modifications for α-La in study **III**. Example ion chromatograms for both of the digested proteins are given in Figure 5. The separation was achieved in a C18 Waters XBridge Prep BEH130 (5 µm, 250 × 10 mm) column attached to a Waters semi-preparative HPLC (Waters 2545 binary gradient module, Waters system fluidic organizer, and Waters 2767 sample manager). The injection volume was 950 µL and the flow rate was originally 6 mL/min (study **I**), but was later scaled down to 5 mL/min (study **III**). An active flow splitter was used to divide in ratio of 1:200 between the detectors and the fraction collector. The detectors included both a single quadrupole mass spectrometer (Waters) and a Waters 2996 PDA. The fraction collection was operated via *m/z*-value trigger recognition. The whole instrument was controlled by Waters MassLynx v4.1 with FractionLynx program. All LC–MS systems developed in the studies are summarized in Table 5.

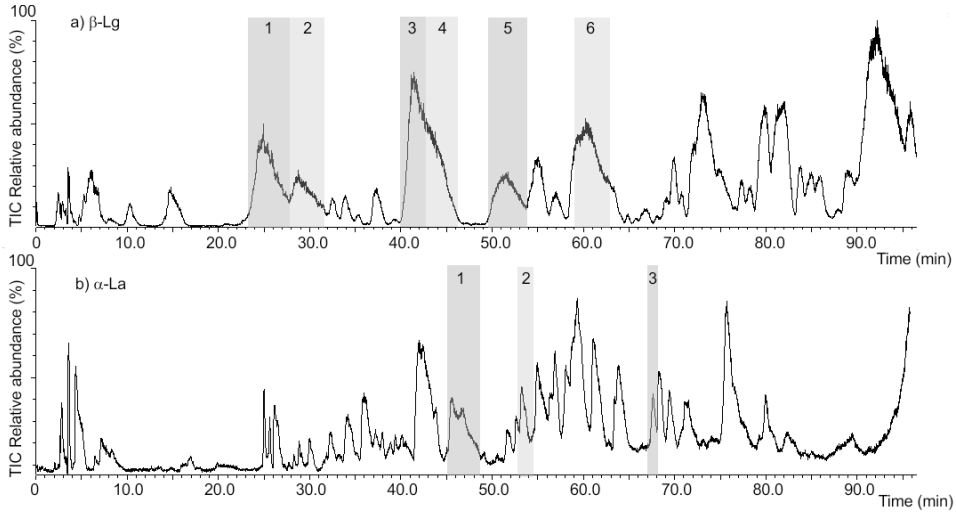


Figure 5. Typical prep-HPLC-MS ion chromatograms for fractionation of the tryptically digested peptides from a) β-Lg and b) α-La. The dominating peptides per highlighted fraction were: a) 1. IDALNENK (*m/z* 917); 2. GLDIQK (*m/z* 673); 3. ALPMHIR (*m/z* 838); 4. LIVTQTMK (*m/z* 934); 5. IPAVFK (*m/z* 674); 6. VLVLDTDYK (*m/z* 1066) all from β-Lg, and b) 1. VGINYWLAHK (*m/z* 1201); 2. LDQWLCEK (*m/z* 1034); 3. W^{+16}LAHKALC (*m/z* 958) all from α-La.

Table 5. The LC–MS systems developed for the studies.

ID	Solvents	Flow rate	Gradient program			Temp.	Detectors
Prep-HPLC for β-Lg Studies **I-II**	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	6 mL/min (5 mL/min)	0 min 15 min 135 min 137 min 142 min	95% A 95% A 65% A 25% A 25% A	5% B 5% B 35% B 75% B 75% B	30 °C	ZQ (*m/z* 100-2000), PDA (190-400 nm)
Prep-HPLC for α-La Study **III**	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	5 mL/min	0 min 15 min 25 min 97 min 97.1 min 102 min 107 min	95% A 95% A 88% A 65% A 25% A 25% A 95% A	5% B 5% B 12% B 35% B 75% B 75% B 5% B	30 °C	ZQ (*m/z* 100-2000), PDA (190-400 nm)
LC–QIT-MS Studies **I-II** Grad.1 (general)	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	0.350 mL/min	0 min 4 min 29 min 35 min 36 min 41 min 42 min	95% A 95% A 84% A 78% A 20% A 20% A 95% A	5% B 5 % B 16% B 22% B 80% B 80% B 5% B	30 °C	DAD (214 nm), FLD (Ex./Em. 280/350 nm), MS^n (*m/z* 200-2200)
LC–QIT-MS Study **I** Grad.2 (special)	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	0.350 mL/min	0 min 2 min 33 min 39 min 40 min 45 min 46 min	95% A 95% A 80% A 74% A 20% A 20% A 95% A	5% B 5% B 20% B 26% B 80% B 80% B 5% B	30 °C	DAD (214, 235, 274 nm), FLD (Ex./Em. 280/350 nm), MS^n (*m/z* 200-2200)
LC–QIT-MS Study **III** Grad.3 (general)	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	0.350 mL/min	0 min 2 min 22 min 30 min 31 min 36 min 37 min	95% A 95% A 60% A 40% A 20% A 20% A 95% A	5% B 5% B 40% B 60% B 80% B 80% B 5% B	30 °C	DAD (214, 260 nm), FLD (Ex./Em. 280/330 nm), MS^n (*m/z* 200-2200)

4.4 Identification of the peptides and location in the sequence

With the tryptic digestion conditions used, the peptide profile of β-Lg consisted of 10 fragments with no missed cleavages and 9 fragments with one missed cleavage (collected range *m/z* 500-2000). Altogether 8 of these peptides were fractionated and identification confirmed with the LC–MS^n, which was preferred instead of direct infusion MS^n. In addition, oxidation pre-testing was carried out with all of these 8 peptides. Finally, three promising peptides were chosen to be oxidized for study **I**, and one additional for study **II**, based on their amino acid sequences of oxidative interest. These peptides, summarized in Table 6, also had good chromatographic separation and were formed high in abundance. One identified β-Lg peptide was also chosen as an analytical in-house reference (described in 4.6.2). The other identified, but not published, peptides were GLDIQK (*m/z* 673), IDALNENK (*m/z* 917), and VLVLDTDYKK (*m/z* 1194).

For α-La, the digestion conditions used produced 5 distinguished peptides with 0 missed cleavages and 2 peptides with one missed cleavage (collected range *m/z* 500-2000), all of which were further fractionated and identified with the LC–MS^n. In addition, one non-typical tryptic digestion product was seen to form in high abundance, and was added to the experimentation (peptide W^{+16}LAHKALC, study **III**). The final chosen peptides are included in Table 6. The additional identified and experimented peptides were EQLTK (*m/z* 618), ALCSEK (*m/z* 650), CEVFR (*m/z* 653), CEVFRELK (*m/z* 1024), as well as LDQWLCEKL (*m/z* 1148).

Table 6. The tryptic peptides fractionated, oxidized and investigated in the studies, presented in the order of their sequence location in each whey protein.

Peptide identification	Location[a]	Observed $[M + H]^{1+}$	Amino acid sequence	Study
β-Lactoglobulin				
LIVTQTMK	1-8 (17-24)	*m/z* 934	Leu-Ile-Val-Thr-Gln-Thr-Met-Lys	I, II
GLDIQK	9-14 (25-30)	*m/z* 673	Gly-Leu-Asp-Ile-Gln-Lys	u. r.
IPAVFK	78-83 (94-99)	*m/z* 674	Ile-Pro-Ala-Val-Phe-Lys	II
IDALNENK	84-91 (100-107)	*m/z* 917	Ile-Asp-Ala-Leu-Asn-Glu-Asn-Lys	u. r.
VLVLDTDYK	92-100 (108-116)	*m/z* 1066	Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys	I
TPEVDDEALEK	125-135 (141-151)	*m/z* 1246	Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-Lys	I, II, III
ALPMHIR	142-148 (158-164)	*m/z* 838	Ala-Leu-Pro-Met-His-Ile-Arg	I, II
α-Lactalbumin				
VGINYWLAHK	99-108 (118-127)	*m/z* 1201	Val-Gly-Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys	III
W^{+16}LAHKALC	104-111 (123-130)	*m/z* 958[b]	Trp^{+16}-Leu-Ala-His-Lys-Ala-Leu-Cys	III
LDQWLCEK	115-122 (134-141)	*m/z* 1034	Leu-Asp-Gln-Trp-Leu-Cys-Glu-Lys	III

[a] Without and with the signal peptides (α-La 1-19; β-Lg 1-16).

[b] $[M + H]^{1+}$ was not the observed dominating form of molecular ion.

u. r. Unpublished results presented first time in this dissertation study.

4.5 Peptide oxidation protocol

4.5.1 Preparation of the concentrated peptide sample

Purities of the collected peptide fractions were evaluated from total ion chromatograms (Prep-HPLC with Waters MassLynx 4.1), and 15 similar fractions were chosen to be combined for each peptide (e.g. 15 × fraction 3: ALPMHIR). Concentration of the combined peptide sample was achieved using a rotary evaporator, with warm water bath not exceeding 30 °C so that no changes in the peptides would be promoted. Acetonitrile (90-100%) was used for rinsing all storage tubes, and when changing from the large round bottom flask to a small pear bottom flask to finish up the evaporation in a suitable scale for continuing the experiment.

4.5.2 Oxidation experiment

Fresh buffer and reagent solutions were always made on the day the oxidation experiment was started; 15 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and 0.1 mM ascorbic acid (pH adjusted to 6.0), with 10 µM $FeCl_3$ (oxidation solution) was used. The concentration of

H_2O_2 was checked with spectrophotometer prior to preparing the working solution. The dry and concentrated peptide sample was soluted into 15 mL of the oxidation solution. After careful soluting (including sonication), the sample was divided into triplicate, and the H_2O_2 was added (1 mM final concentration). The closed sample tubes were placed randomly in an oven at 37 °C. Sub-sampling was obtained on oxidation days 0, 1, 4, 7, 10, and 14.

In study **II**, evolution of oxidation of the chosen peptides was monitored within time also in the presence of an added phenolic component, Sanguiin H-6. The phenolic-to-peptide ratio was 1000 µg of sanguiin H-6 per 1 g of each peptide (molar ratio of 1:2000).

4.6 LC–MS investigations

4.6.1 Analysis of the peptides and peptide oxidation products

In all studies (**I-III**) the main HPLC instrument used was the Agilent 1100 system (Agilent Technologies, Santa Clara, CA, U.S.A.) involving a binary pump, degasser, sample manager, column heater, diode array detector and fluorescence detector. Separation was achieved with Waters XBridge BEH130 C18 column (2.1 × 100 mm, 3.5 µm), which was specially designed for peptide separation, and including a guard column of the same material. Injection volume of 10 µL was typically used and the different gradient elution conditions are presented in Table 5. All in all, the gradient programs were used as follows: Grad.1 for all the β-Lg peptides, except Grad.2 for peptide VLVLDTDYK (*m/z* 1066), and Grad.3 for all of the α-La peptides presented.

Recorded DAD ranged from 180-400 nm, but λ 214 nm was generally used, with single wavelengths 235, 260 and 274 nm analyzed for additional information, when applicable. Fluorescence detector was operated mainly with 280/350 nm (excitation/emission).

This HPLC-setup was directly connected to the Bruker Esquire quadrupole ion trap mass spectrometer (Bremen, Germany) with electrospray ionization (QIT-ESI-MS). Optimization of the MS-parameters was carefully performed; in quadrupole ion trap it is possible to tune multiple parameters, and different values may favor shorter and longer peptides. After screening with several peptides, the peptide APMHIR (*m/z* 838) was chosen as the reference for final optimization. Positive mode was preferred (studies **I-III**), and the attuned parameters were: dry gas 9.0 L/min, nebulizer 70.0 psi, ESI interface dry temperature 300 °C, capillary voltage 4.5 kV, end-plate offset -400 V, skimmer 1 65.0 V, skimmer 2 20.0 V, lens 1 -2.0 V, lens 2 -50.0 V, octopole 4.5 V, octopole delta 2.7 V, capillary exit 200.0 V, capillary exit offset 135.0 V, and trap drive value 80.0. Fragmentation amplitude was adjusted according to the individual need (e.g. stage of MS^n), but generally the value of 1.45 was set for MS^2.

For study **II**, optimization of the negative mode MS was required. All 3 peptides (ALPMHIR, IPAVFK, and LIVTQTMK) were used in the optimization process and finally the following MS parameters were chosen: dry gas 8.0 L/min, nebulizer 60.0 psi, ESI interface dry temperature 300 °C, capillary voltage 3.8 kV, end-plate offset -500 V, lens 1 5.0 V, lens 2 60.0 V, capillary exit -178.5 V, and trap drive value 102.5.

The LC–MS data was analyzed using the Agilent ChemStation and LC/MSD Trap 5.2 programs, from where data was exported to Microsoft Excel. Identification of the peptides and their oxidation products was based primarily on the fragmentation information gathered. In addition to own structure based calculations, the Universal Protein Resource databases (UniProt), the comprehensive database of protein modifications for mass spectrometry (Unimod; Creasy and Cottrell 2004) and the Bioinformatics Resource Portal of the Swiss Institute of Bioinformatics (ExPASy) were explored as trusted references.

4.6.2 Preparation and use of the in-house reference sample

The β-Lg peptide TPEVDDEALEK with *m/z* 1246 was fractionated, collected and concentrated according to the above presented protocol (4.2-4.5.1). However, instead of using the oxidation reagent solution, the dried sample was soluted in 15 mM PIPES (pH 6.0) and divided into aliquots in multiple ready-to-use sample vials. These were stored in -20 °C until further use.

In each sample batch analyzed with the MS, at least one fresh vial of the in-house reference sample was defrosted and used. The in-house reference peptide was always analyzed in the beginning of the sample set, and with the "General gradient 1" (Table 5). Integration was performed with same processing parameters as for all the other compounds, and from the EIC's of ion *m/z* 1246.

Originally, multiple analyses of peptide TPEVDDEALEK were carried out during several injection days and a reference table was set, with action limits based on mean ± 2 × standard deviation (SD). If the reference peptide resulted in a value outside the action limits, the whole sample set was rejected and re-analyzed (instrumentation not considered reliable enough). The repeating analyses of the reference peptide also revealed that results within the same sample set varied less than between different days. Therefore, all sub-samples belonging to the same investigated peptide were always run together in the same batch to minimize day-to-day variance, with one exception in study **III**. Due to instrumental challenges, the sub-samples from oxidation days 0 and 1 of peptide VGINYWLAHK had to be re-analyzed on a different day than rest of this peptide batch. A slight day-to-day change in overall intensity was observed between the two sets, based on the reference peptide comparisons, and although both fit inside the action limits, this resulted in a likely overestimation of oxidation products when presented in the same figure. Therefore, a conversion factor was calculated based on the reference peptide area results, and the values were corrected for the day 0 and 1 sub-samples, whereas the values were original for the day 4-14 sub-samples. No conversion factors were calculated, nor used, for any of the other peptides studied.

5 RESULTS

5.1 Method development

5.1.1 Identification and investigation of the in-house reference peptide

The peptide TPEVDDEALEK, seen with the molecular ion $[M + H]^{1+}$ of *m/z* 1246, was found to be one of the major tryptic digestion products of β-Lg. However, it did not show to undergo any significant MCO reactions in the pre-studies. The sequence of the intact peptide was confirmed by LC–MS/MS and the resulting fragment ions are presented in Table 7. Due to the observed stability and available abundancy, the peptide TPEVDDEALEK was chosen to be fractionated for the use as an in-house reference peptide.

Table 7. Fragmentation observed for the peptide TPEVDDEALEK, $[M + H]^{1+}$ *m/z* 1246. This peptide was used as an in-house reference in all MS analyses. The most intense fragment ions are indicated in boldface (relative intensity > 75%).

m/z	Fragment identification	Notes
1144	PEVDDEALEK	
1099	TPEVDDEALE $-H_2O$	Water likely lost from Thr side chain due to formation of C=C
1047	EVDDEALEK	
970	TPEVDDEAL $-H_2O$	Water likely lost from Thr side chain due to formation of C=C
918	VDDEALEK	
857	TPEVDDEA $-H_2O$	Water likely lost from Thr side chain due to formation of C=C
819	DDEALEK	
786	TPEVDDE $-H_2O$	Water likely lost from Thr side chain due to formation of C=C
756	TPEVDDE $-H_2O$ $-2O$ $+2H$ *or* TPEVDDE $-H_2O$ $-CH_3$-CH_3	Further fragmentation of *m/z* 786 with loss of 2O (Asp or Glu) and rearrangement with +2H *or* by further Thr breakage with loss of CH_3-CH_3
704	DEALEK	
657	TPEVDD $-H_2O$	Water likely lost from Thr side chain due to formation of C=C
589	EALEK	
556	EALEK –OOH	Loss of –OOH ($2H_2O$) from Glu, with formation of a C=C
460	ALEK	
389	LEK	
326	TPE $-H_2O$ $-H_2$	Water likely lost from Thr side chain due to formation of C=C, and additional H_2 abstraction

5.1.2 Identification of peptide W^{+16}LAHKALC

The peptide W^{+16}LAHKALC was found to be an abundant compound in the preparative-HPLC chromatograms of the α-La peptides, although it was not a typical and expected tryptic hydrolysis product. However, it was a match to the ExPASy FindPept results of an unspecific cleavage located in the sequence position 104-111 (123-130). The peptide was fractionated with prep-HPLC and identified by LC–MS^n fragmentation with varying amplitudes. The identified fragments of the protonated molecular ion *m/z* 958, the $[W^{+16}LAHKALC + H]^{1+}$ are listed in Table 8. With the MS-instrument used, the intensities of the fragment ions were greatly influenced by the fragmentation parameters used, and loss of side chains was observed very typical. The named ions with two identifications were most likely a sum of both, as for example the protonated peptides HKA and AHK resulted in same mass-to-charge ratio ions (Table 8).

Table 8. Fragmentation observed for peptide W^{+16}LAHKALC, from the molecular ion $[M + H]^{1+}$ *m/z* 958. The most intense fragment ions are indicated in boldface (relative intensity > 75%).

m/z	Fragment identification	Notes
940	W^{+16}LAHKALC $–H_2O/NH_4$	
912	W^{+16}LAHKALC –CSH	Loss of Cys side chain
886	W^{+16}LAHKALC –CSH –NC	Breakage of His (loss of N=C)
868	W^{+16}LAHKALC –CSH –NC $–H_2O/NH_4$	
840	W^{+16}LAHKAL	Loss of Cys, with C-term. $–CH_2OH$ instead of –CHO
773	*m/z* 755 + 18	Possibly with H_2O
755	LAHKALC	Loss of Trp^{+16}
727	W^{+16}LAHKA	Loss Leu, with C-term. $–CH_2OH$ instead of –CHO
671	W^{+16}LAHK	Loss of Ala
587	-	Not identified, possibly either ion below with additional –OH
574	HKALC +2H	Double bond opening
570	HKALC –2H	Double bond formation
541	LAHKA & AHKAL	Sum of both, with $–CHOH–NH_2$ instead of $–CO–NH_2$ on C-term.
424	AHKA	
406	AHKA $–NH_4$	Loss of the C-term. amino group and double bond rearrangement
378	AHKA $–NH_4$ –CO	Further cleavage from C-term.
361	AHKA $–NH_4$ –CO –OH	Further cleavage
335	HKA $–H_2O/NH_4$ & AHK $–H_2O/NH_4$	Sum of both

Some confusion was experienced due to an ion of *m/z* 936 eluting in the same preparative fraction with *m/z* 958. The two ions were first considered to have some relation to each other, due to the mass difference of only 22 u that may have indicated for example a sodium adduct. In comparison of the fragmentation patterns of these two ions, presented in Figure 6, no relationship could be confirmed. However, the ion *m/z* 936 was the dominant ion for this fraction at the preparative-HPLC, which is why it was originally used as the mass trigger of the fraction collector. With the LC–QIT-MS the ion *m/z* 958 was the dominant ion seen in the analyzed fraction, and resulted in the identification presented in Table 8.

Relatively strong sodium adduct formation was observed for this peptide, and in some oxidation sub-samples the $[M + H]^{1+}$ (*m/z* 958) was clearly dominated by $[M + Na]^{1+}$ (*m/z* 980). As discussed in study **III**, this observation led to the decision to monitor this peptide as the sum of these two molecular ion forms. The same trend was equally pronounced for the oxidation product compound ions (Figure 7), which were also monitored as a sum of the two ions; $[M_{ox} + H]^{1+}$ and $[M_{ox} + Na]^{1+}$.

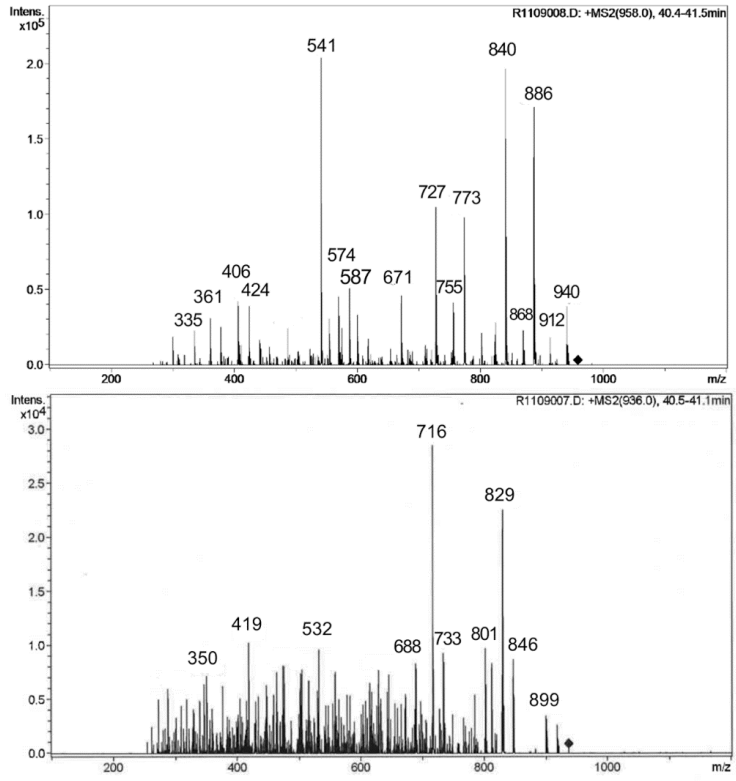


Figure 6. Comparison of MS^2-fragmentation of the two main ions collected in the same Prep-HPLC chromatographic fraction, the mother ion *m/z* 958 (on top) and the mother ion *m/z* 936 (below). The main fragment ions of *m/z* 958, the peptide $[W^{+16}LAHKALC + H]^{1+}$, are identified in Table 8.

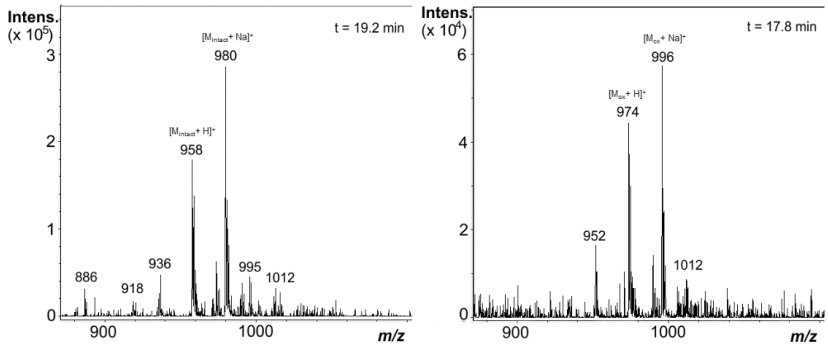


Figure 7. The observed molecular ions $[M + H]^{1+}$ and $[M + Na]^{1+}$ of the intact peptide W^{+16}LAHKALC (left) and its oxidation product with +16 u (right). In the MS-analyses both peptides showed the same strong sodium adduct formation trend as well as protonation, and therefore results are presented as a sum of both major ions.

5.1.3 Identification of other peptides studied

None of the studied peptides showed any significant tendency to multiply charged ions. Sodium adduct formation was observed for several of the peptides and/or their oxidation products, yet very moderate compared to the favored protonation, except for the already mentioned special case; the W^{+16}LAHKALC. All of the peptides isolated, collected and oxidized in the experiments, and mentioned in Table 6., were confirmed to match their theoretical expected sequences in a similar way to the before mentioned peptides TPEVDDEALEK and W^{+16}LAHKALC, reported in sections 5.1.1 and 5.1.2, respectively.

Fragmentation with the LC–ESI-QIT-MS^n did not always favor peptide backbone cleavage, thus consistent side chain fragmentation was also observed. In many cases oxidation of a side chain increased the probability of its fragmentation. The ion trap MS parameters were adjusted according to individual needs and enough fragmentation of peptide backbone was achieved for all peptides to be reliably identified. Two examples of the peptide identification practice are given below, with Figure 8A showing the fragmentation pattern of VLVLDTDYK and Figure 8B the fragmentation of peptide IDALNENK. The named cleavages included several typical losses of H_2O (-18 u) and $–NH_3$ (-17 u). In Figure 8A an example of a typical side chain fragmentation was observed as the subsequent ion *m/z* 806, which corresponded to the further cleavage of fragment VLVLDTDY (*m/z* 919) by loss of H_2O (to *m/z* 901), followed by loss of the phenyl moiety of the current C-terminal amino acid Tyr. In the rearrangement process a new double bond was formed (thus resulting in *m/z* 806). In Figure 8B additional side chain fragmentation was observed for example via further oxygen cleavages from the acidic side chain of Glu in peptide fragment ENK.

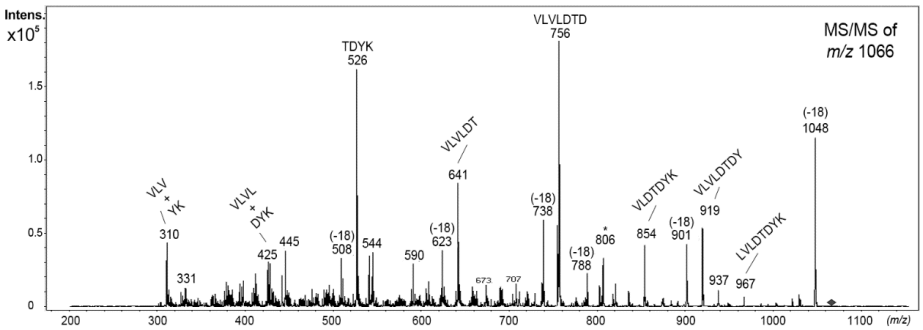


Figure 8A. Typical identification example via MS/MS fragmentation, the β-Lg peptide VLVLDTDYK fragmented from the molecular ion $[M + H]^{1+}$ *m/z* 1066. The cleavages of -18 u correspond to losses of H_2O. The ion *m/z* 806 marked with * is the result of observed typical side chain fragmentation tendency, here resulting from the peptide fragment VLVLDTDY with additional cleavages of H_2O, the phenyl side chain moiety of Tyr, and formation of a double bond.

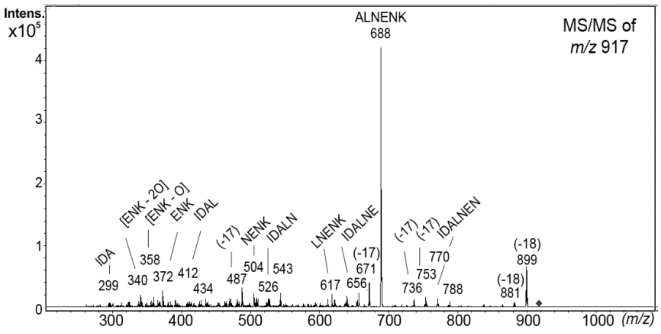


Figure 8B. Typical identification example via MS/MS fragmentation, the β-Lg peptide IDALNENK fragmented from the molecular ion $[M + H]^{1+}$ *m/z* 917. The cleavages of -18 u correspond to losses of H_2O and cleavages of -17 u to losses of amino groups ($-NH_3$). In addition, the acidic side chain of Glu in peptide fragment ENK preferred fragmentation of both of the oxygens of –COOH.

5.2 Side chain amino acid MCO reactions observed

An array of oxidation modifications was witnessed for the studied peptides. Most of the new peptide products were nicely separated in chromatography from their intact peptide form. Additional information from the other detector tools available besides MS were also used in the identification of the new products, e.g. loss of fluorescent properties or changes in absorbance on wavelengths typical for aromatic compounds.

In a number of peptides, oxidation produced two or more chromatographic peaks that resulted in the exact same ion. In these cases closer identification of the modified moieties were attempted by analysis of their fragmentation. A successful example is presented in Figure 9, where primary oxidation of the β-Lg peptide ALPMHIR (*m/z* 838) produced two closely eluting (± 0.5 min) compounds with the exact same ion (*m/z* 854), yet different behavior in further reactivity within time. In their fragmentation profiles (study **I**) both mother ions even produced the same main fragment (*m*) of *m/z* 790, but the critical difference was between their isotopic *m*+1 and *m*+2 ions. For compound A only *m*+1 (*m/z* 791) was seen and for compound B the fragmentation had not destroyed the sulphur-containing Met side chain so also *m*+2 ion (*m/z* 792) was recorded. Furthermore, compound A was assigned to peptide ALPM^{+16}HIR, where the fragment ion *m/z* 790 resulted from the characteristic loss of the sulfoxide side chain (Δ 64 u, CH_3SOH). Compound B could not be confirmed to full certainty, but one likely match was the peptide ALPMH^{+16}IR having 2-oxo-His. In this case the ion *m/z* 790 resulted from the typical carbonyl loss of 28 u together with 2 × H_2O (Δ 18 u each). Likewise, the Δ 28 u could have been cleaved from the Pro residue that was oxidized to a GGS. In study **II**, the amount of both compounds formed by oxidation, with the ions *m/z* 854, was so high that it was eventually decided to integrate both chromatographic peaks together as one product to monitor.

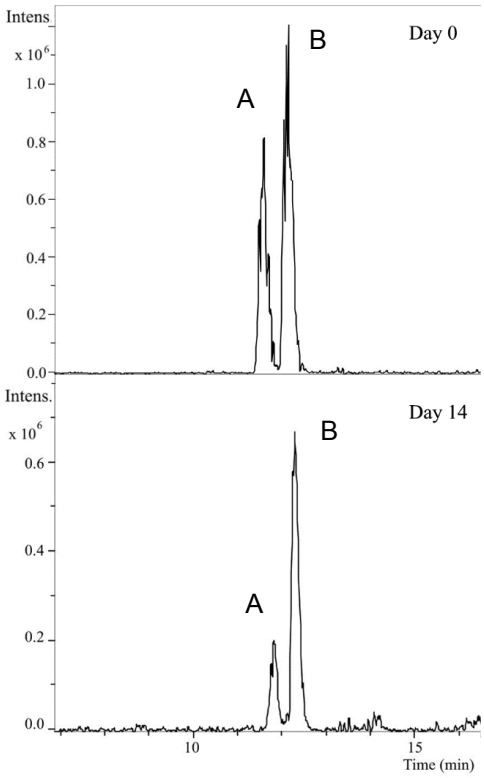


Figure 9. Extracted ion chromatograms of *m/z* 854, showing both of the two main oxidation products from β-Lg peptide ALPMHIR throughout the time of experiment (adapted from Study **I**). Fragmentation confirmed compound A to result from Met oxidation, whereas compound B had intact Met, but either His or Pro oxidized.

5.2.1 Methionine oxidation

Met was found highly prone to oxidize in both of the two studied peptides that contained Met. In the β-Lg peptide ALPMHIR, oxidation of Met was so rapid that it clearly dominated over the intact peptide already on oxidation day 0. Two separate oxidation products with the same ion *m/z* 854 were observed, as discussed in section 5.1 above with Figure 9, of which the first-eluting peak was the confirmed result of Met oxidation to Met sulfoxide. In addition, a third oxidation product compound with its own chromatographic peak was seen with the ion *m/z* 970. The formation trend of this modified peptide matched quite well with the loss of peptide [ALPM^{+16}HIR], and it was postulated that oxidation of the Met residue continued all the way to Met sulfone form, the [ALPM^{+32}HIR] (*m/z* 838 + 32 u = *m/z* 970). By the end of the oxidation experiment, this compound had become the dominating peptide found in the samples.

Also in the β-Lg peptide LIVTQTMK the primary oxidation to Met sulfoxide was eminent. In fact, compared to the amount of intact peptide found, more than 10-fold of the intact peptide

was oxidized to [LIVTQTM^{+16}K] already on day 0. The amount of this oxidized peptide, monitored with the ion *m/z* 950, remained high throughout the whole 14-day experiment. An additional oxidation product [LIVTQTMK + 2O] was observed forming especially between the days 1-4, and seen with the ion *m/z* 966 (*m/z* 934 + 32 u), which most likely resulted from Met dioxidation to Met sulfone. This hypothesis was in line with the fact that some reversibility was observed for [LIVTQTM^{+16}K]; its amount was decreasing between days 1-7 and then slightly turned to the rise again between days 7-14.

Interestingly in study **II**, very high amount of peptide [LIVTQTMK + 2O] was detected in the samples without the added phenolic compound Sanguiin H-6, already since day 0. In the parallel peptide samples with Sanguiin H-6, the formation of this dioxidation was very modest. It also seemed that Sanguiin H-6 could serve as an antioxidant towards overall Met oxidation in this peptide, and the effect was significant especially from day 4 onwards. For peptide ALPMHIR the addition of Sanguiin H-6 did not have a significant antioxidant effect against Met – in fact it may be that the influence was slightly pro-oxidant in time, again from 4th day onwards. The potential interaction product formation between the peptide LIVTQTMK and Sanguiin H-6, possibly through Lys (rather than Met), has been further considered in the discussion of study **II**.

5.2.2 Cysteine oxidation

Cysteine was part of the sequences of two of the studied peptides, both being α-La derived (study **III**). In peptide LDQWLCEK (*m/z* 1034), a rapid and significant formation trend of observed ion *m/z* 1082 was witnessed within the first 24 h. Cys was named as the primary target site, and the modification matched Cys trioxidation all the way to Cys sulfonic acid, the peptide [LDQWLC^{+48}EK]. In addition, corresponding rapid loss of the intact peptide was noted. However, the evolution of ion *m/z* 1082 soon turned to a declining trend, and secondary oxidation reactions were observed to form from the peptide [LDQWLC^{+48}EK], seen as the rise of two additional compounds with ions *m/z* 1098 (*m/z* 1082 + 16 u). These two new oxidation products were not designated to include any further Cys modification, but had changes in other amino acid side chains. In the other α-La peptide, W^{+16}LAHKALC, Cys oxidation was reflected but not reported in the published studies.

5.2.3 Histidine, proline and arginine oxidation

In β-Lg peptide ALPMHIR, one oxidation product of +16 u was observed in addition to the confirmed Met modifications. The MS^n-fragmentation could not define the exact placement of the oxygen molecule in the amino acid side chains, but either His or Pro was considered as most likely target sites. Both of them are known to oxidize into a carbonyl compound; His to 2-oxo-His and Pro to γ-glutamic semialdehyde (GGS). In addition, GGS is the known primary oxidation product of Arg, also present in peptide ALPMHIR. Even though the oxidation product [ALPMHIR + O] could not be named to full certainty, it was noted that only one seemed to be forming. This was also a very significant oxidation product of peptide

ALPMHIR, and was very easily monitored with the ion *m/z* 854 throughout the whole 2-week oxidation experiment. The formation trend of this peptide [ALPMHIR + O] saw a decreasing trend towards day 4, suggesting secondary oxidations to the same peptide, but after that remained rather level or even slightly increasing again.

The α-La peptide W^{+16}LAHKALC produced a number of oxidized peptides by different amino acid side chain modifications, but only one relatively stable product that could potentially be used for monitoring. The exact oxidation site could not be confirmed, but His modification to 2-oxo-His was considered a very prospective end product. Unfortunately the low intensity of the fragment ions could not confirm the characteristic fragmentation of 2-oxo His, the loss of Δ 28 u (C=O). However, resemblances to the formation trend of the β-Lg derived [ALPMHIR + O], also suspected to be 2-oxo-His, were noted.

In addition, similar unidentified but rather significant primary oxidation product was observed from peptide VGINYWLAHK, the one overlapping in α-La sequence with W^{+16}LAHKALC. Also for VGINYWLAHK the formation of 2-oxo-His was strongly considered, although other possibilities could not be ruled out in efforts to confirm by fragmentation. Furthermore, formation of the presumed 2-oxo-His was not the dominating oxidation product in either of the α-La peptides named.

Therefore, the observations of possible His oxidation in both α-La and β-Lg peptides include the hypothesis that if His is oxidized, and producing 2-oxo-His, the product seems not to be eager to continue with further oxidation. Therefore 2-oxo-His might result in a stable oxidation marker for monitoring purposes.

The peptides IPAVFK and TPEVDDEALEK included in the studies did not produce substantial Pro oxidation to be reported.

5.2.4 Lysine oxidation

Substantial Lys oxidation to its known major oxidation product, the α-aminoadipic semialdehyde (AAS), was reported in significance only for the α-La peptide VGINYWLAHK. In this peptide the AAS modification dominated by both the rate of development and as the single product with highest total amount formed compared to the array of other oxidation products detected, including the individual Trp modifications (see section 5.2.7, Figure 13). However, as a sum, more Trp oxidation products were formed than AAS from Lys, as demonstrated in Figure 14. Nonetheless, the abundant new protonated molecular ion *m/z* 1200 (Δ -1 u) was easily monitored throughout the whole 14-day experiment, and especially as the intact peptide and the [VGINYWLAHK-AAS] eluted 5 min apart from each other, in this order. Both the chromatographic separation and the mass spectral differences are portrayed in Figure 10.

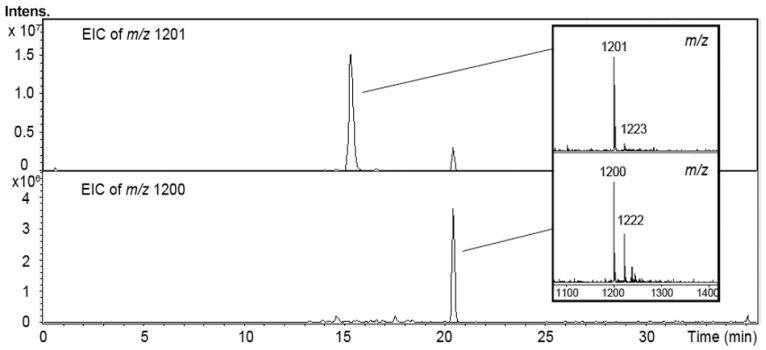


Figure 10. Two single ion chromatograms from the oxidation investigation of α-La peptide VGINYWLAHK. Above, EIC of *m/z* 1201 corresponding to the intact peptide eluting at t_R 15.5 min. Below, EIC of *m/z* 1200 corresponding to the peptide where Lys has oxidized into the α-aminoadipic semialdehyde form, thus eluting notably later at t_R 20.5 min.

For the other α-La peptide ending with Lys, the peptide LDQWLCEK, no AAS formation was detected. Here, Cys was the most prone amino acid to oxidize, with also the secondary reactions attributed to other amino acids than Lys and seen via further mass additions of +16 u. Likewise, no Lys oxidation was observed for the β-Lg peptide LIVTQTMK, as other amino acid side chains reactions, mainly Met, were found to be preferred. Also in peptide VLVLDTDYK no Lys oxidation was reported in study **I**, but it was in fact hypothesized that 'oxidation product B' (Figure 7. in study **I**) could be resulting from a rather slow Lys oxidation to AAS.

Previously unpublished results of the additional β-Lg peptides GLDIQK and IDALNENK revealed that Lys oxidation to AAS was an excellent target product for monitoring of their oxidation status. In IDALNENK, the unoxidized peptide was seen with $[M + H]^{1+}$ *m/z* 917, whereas the AAS-peptide was monitored by the ion *m/z* 916. The formation of the AAS-peptide was markedly increasing in time, as portrayed in Figure 11a. In GLDIQK the unoxidized peptide was seen with $[M + H]^{1+}$ *m/z* 673, while its oxidized AAS-form was observed with the ion *m/z* 672. In this peptide, the formation trend was quite different, as seen in Figure 11b; the maximum notable amount of [GLDIQK-AAS] was reached within the first 24 h of oxidation, and from thereon secondary oxidation reactions in the same semi-aldehydes could be witnessed as a decrease of the ion *m/z* 672 within time. In peptide GLDIQK multiple further oxidation reactions of several other amino acids were observed (data not included), whereas not very significant additional oxidation was documented for peptide IDALNENK.

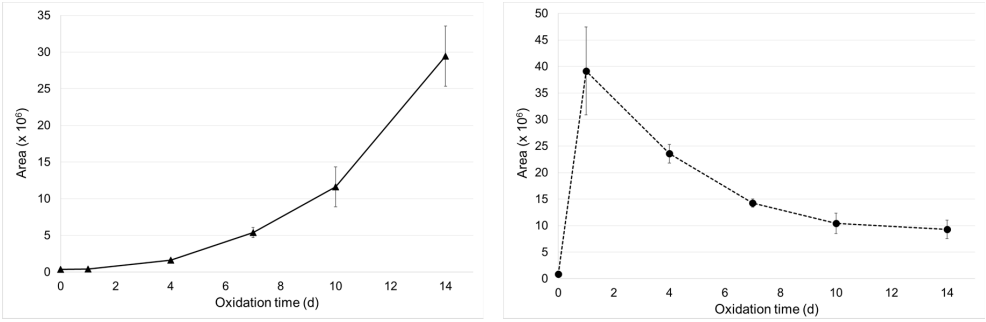


Figure 11. Evolution within time of Lysine oxidation to α-aminoadipic semialdehyde (n = 3). Previously unpublished results of the studied peptide modifications: a) [IDALNENK-AAS] b) [GLDIQK-AAS].

5.2.5 Tyrosine oxidation

Dityrosine (diTyr) formation was found to be the major oxidation product in β-Lg peptide VLVLDTDYK. The amount of the resulting diTyr-dipeptide was quite modest by comparison of the UV and especially the EIC peak area, but the corresponding fluorescence peak of the oxidation product was very prominent, as illustrated in Figure 12. On the other hand, it may be that the diTyr-dipeptide was both singly $[M + H]^{1+}$ and doubly protonated $[2M + 2H]^{2+}$ in the samples, even though only the doubly charged end product could be monitored with the MS instrumentation used. Furthermore, it should be noted that the diTyr-dipeptide was actually observed with the same *m/z* 1066 ion as the intact peptide, instead of the theoretically expected *m/z* 1065, due to decimal issues of the instrumental accuracy (the small amount formed varied slightly between *m/z* 1065.4–1065.7). The expected singly charged diTyr-dipeptide with *m/z* 2129 could unfortunately not be confirmed.

Moreover, Tyr oxidation to 3,4-dihydroxyphenylalanine (DOPA, accounting for +16 u) did not occur at all in peptide VLVLDTDYK. Formation of primary Tyr oxidation to DOPA or diTyr were also investigated from the α-La peptide VGINYWLAHK, but no traces are to be reported.

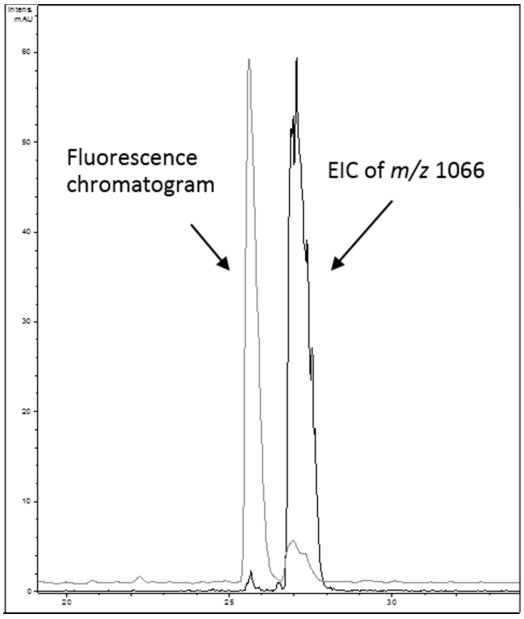


Figure 12. Observations of peptide VLVLDTDYK from oxidation day 14. The first eluting compound (~26 min) showing minor peak area for extracted ion *m/z* 1066, but major response from the fluorescence detector due to diTyr, while the second eluting compound (~27 min) had only minor fluorescent response, yet a major compound by amount (intact peptide).

5.2.6 Phenylalanine oxidation

Phe oxidation was of special interest in the investigations of β-Lg peptide IPAVFK (study **II**). However, no significant Phe oxidation products, such as the typical +16 u and +32 u additions to 2- or 3-hydroxyphenylalanine or Tyr (4-OH-Phe), or further to 2,3-dihydroxyphenylalanine or DOPA (3,4-diOH-Phe), could be declared from the fractionated samples. The total amount of peptide IPAVFK, monitored via the ions *m/z* 674 (positive mode) and *m/z* 672 (negative mode), was not observed to decrease markedly during the 14 day experimentation, and the only recognized oxidation of that peptide was assigned to Lys modification to AAS. Therefore the possible Phe oxidation products remained marginal so that no designations or conclusions could be drawn.

5.2.7 Tryptophan oxidation

Even before the onset of the experimental oxidation study, Trp oxidation was found dominating in the α-La peptide WLAHKALC. The identification of this untypical tryptic hydrolysis product, the actually fractionated monoxidized peptide W^{+16}LAHKALC is discussed in more detail in chapter 5.y. Only one relatively stable oxidation product was observed to form from this peptide, and dioxidation of the Trp moiety was considered as a possible explanation. As the exact position of the OH-groups could not be determined with the methods used, the dioxidation would in theory result in a combination of two of the following isomers: 4-/5-/6- and/or 7-OH-Trp, or possibly from Oia to diOia.

Formation of *N*-fku from the peptide W^{+16}LAHKALC was not observed, as evaluated by the fragmentation of [W^{+16}LAHKALC + O] that did not reveal the loss of NHCHO (Δ 44 u), known as the characteristic fragmentation piece of *N*-fku. On the other hand, *N*-fku is not a direct dioxidation product to be expected from the already characterized W^{+16}.

The peptide W^{+16}LAHKALC was partly overlapping in α-La sequence position with the other studied peptide VGINYWLAHK. In this latter peptide, the oxidation of Trp also produced an array of modifications, all of which could be nicely monitored during the 2-week experimentation. The primary oxidation products, the peptides VGINYW^{+16}LAHK, included two compounds with the same ion *m/z* 1217 and were attributed to Oia and 5-OH-Trp or one of its isomers. Both of them had similar formation trends, with higher amounts during the first experimentation week and decreasing towards and onwards the second week (Figure 13). In addition, three new compounds were detected with another same mass-to-charge -ratio, the ion *m/z* 1233, and all were forming on the later oxidation days. One of these compounds was tentatively identified as a result of diOia formation, although the peptide appeared in very small amounts. Another peptide seemed to be of non-Trp oxidation origin. The compound with a retention time of 12.5 min was, however, a very likely match to *N*-fku formation, including loss of its fluorescent properties (280/330 nm ex./em. used) due to the Trp side chain pyrrole moiety cleavage. The evolution of this modified peptide [VGINYWLAHK/*N*-fku] was quite modest, but the amount formed by the 4th day did not significantly change during the remaining 10 days of the oxidation experiment. The formation of [VGINYWLAHK/*N*-fku] in comparison to the other main oxidation products of the α-La peptide VGINYWLAHK are presented in Figure 13.

In the α-La peptide [LDQW^{+16}LC^{+48}EK], Trp was observed secondary to Cys trioxidation. The findings were supported by loss of fluorescence properties for the compound with ion *m/z* 1098, but not for the ion *m/z* 1082 and the native peptide *m/z* 1034. On the other hand, Trp oxidation to *N*-fku was also investigated, but not observed.

The possibility of diTrp dimeric cross-linking was also investigated for all the peptides containing Trp, but no traces of such modifications were detected. In addition, neither of the two Trp of β-Lg were included in the studied peptides, and the two Trp reported above contribute to 50% of the total Trp content in full α-La protein sequence.

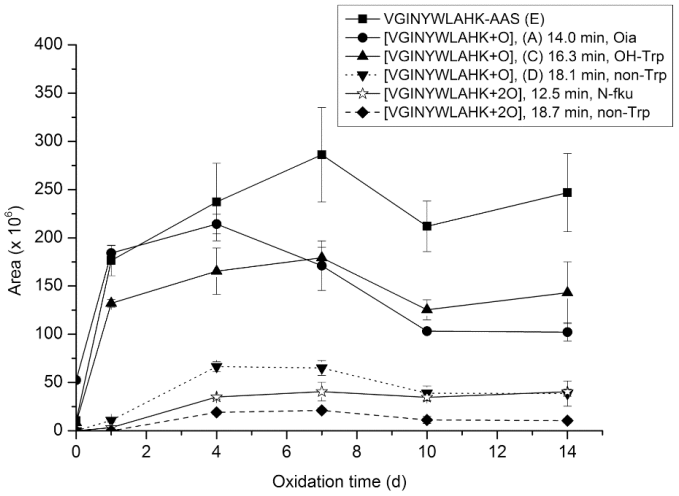


Figure 13. Evolution of the main oxidation products and comparison of the harmful [VGINYWLAHK/*N*-fku] to the others formed from the α-La peptide VGINYWLAHK, as monitored from their extracted ion chromatograms by LC–MS (adapted from study III).

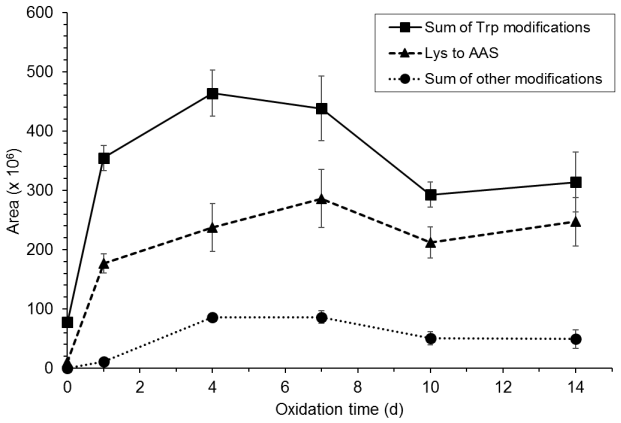


Figure 14. Comparison of contribution of the Lys and Trp residues to the oxidation of peptide VGINYWLAHK.

5.2.8 Further findings of amino acids susceptible to oxidation in the studied peptides

One additional unidentified secondary oxidation modification was detected for the α-La peptide LDQWLCEK, accounting for +16 u. Its formation took place after the Cys trioxidation to sulfonic acid, and was monitored with the ion *m/z* 1098 (*m/z* 1034 + 48 + 16). Asp (D) monoxidation to 3-hydroxyaspartic acid (Hya) was proposed as one possibility (study **III**). Unfortunately, this could not be confirmed by MS^n due to low intensity of the fragment ions. Asp was also present in the β-Lg peptides TPEVDDEALEK, GLDIQK and IDALNENK, none of which showed significant oxidation modifications to report. The same was considered with the other negatively charged amino acid Glu (E). In peptide VLVLDTDYK Tyr was clearly favored over the other amino acids, including Asp, which was not reported to undergo any changes.

Alike to above, both Asn (N) and Gln (Q) have similar side chains with expected similarity to oxidative susceptibility. One unidentified primary oxidation product was detected from the α-La peptide VGINYWLAHK, and monitored with the ion *m/z* 1217 (item D in Figure 13). This new peptide appeared to be of non-Trp origin due to retaining its fluorescent properties and notably later elution from the other oxidation products. Asn oxidation to 3-OH-asparagine was one considered possibility. However, the exact placement of this oxidation site could not be confirmed by fragmentation. In the second studied peptide that included Asn, the IDALNENK, oxidation of this site was not observed. Gln was part of the β-Lg peptides LIVTQTMK and GLDIQK, as well as the α-La peptide LDQWLCEK, but no Gln oxidation was observed in any of them.

Val oxidation to 4-hydroxyvaline was considered as the other possibility for the ion *m/z* 1217 from the α-La peptide VGINYWLAHK. Val oxidation instead of Asn may have been favored due to being the terminal amino acid in the peptide, and it was unfortunate that this could not be confirmed to full certainty. On the other hand Val was not reported a significant oxidation site in peptides LIVTQTMK or VLVLDTDYK.

Furthermore, one secondary oxidation product arising from the α-La peptide LDQWLCEK was postulated as a possible result of Leu oxidation to either 3-, 4- or 5-hydroxyleucine. Further observations include that Leu was also present in VGINYWLAHK, WLAHKALC as well as in ALPMHIR, LIVTQTMK, TPEVDDEALEK, VLVLDTDYK, GLDIQK, IDALNENK, with no assigned oxidative reactivity. Also Ile included in many of the peptides was not found to be prone to oxidize.

5.3 The confirmed and considered susceptible oxidation sites in α-La and β-Lg

According to the findings reported in this dissertation study, the side chains of several amino acids were noted and confirmed to be the highly susceptible target sites for MCO. From the α-La peptide studies, the confirmed oxidation sites were Trp104, Lys108, Trp118, Cys120, and most likely, but not fully confirmed sites His107, and Cys111. From the β-Lg peptide studies the confirmed main targets were Met7, Lys14, Lys91, Tyr99, Met145 and additional suspected sites Lys100, as well as either Pro144 or His146 (only one in addition to the confirmed Met145 in the middle).

Furthermore, the studies confirmed a couple of suspected amino acid sites to not have undergone significant modification from MCO. Despite of being the terminal amino acid in peptide LDQWLCEK, Lys122 was not oxidized to AAS. In addition, no diTyr formation was detected from Tyr103 in peptide VGINYWLAHK. In β-Lg both Lys8 and Arg148 did not show oxidation modifications even though they were both the C-terminal amino acids of their respective peptides.

5.4 The proposed protein oxidation marker peptides

The most potential oxidation marker peptides were considered based on two criteria; susceptibility to oxidize as well as stability of the formed peptide product for monitoring purposes. The proposed promising marker peptides are summarized in Table 9. Several other oxidation products were also detected, but in most cases they were too prone for further reactions to be used to reflect the overall status of any whey food product. In addition to those listed in Table 9. the diTyr-dipeptide VLVLDTDYK (observed with the ion *m/z* 1066 as the doubly charged $[2M + 2H]^{2+}$ molecule) is proposed as a promising oxidation marker for monitoring purposes either by MS or fluorescence detection.

The promising indicators of β-Lg oxidation, that could be used as marker peptides for monitoring purposes were the ions *m/z* 854 corresponding to [ALPMHIR + O], *m/z* 870 to [ALPMHIR + 2O], *m/z* 950 to [LIVTQTMK + O], and *m/z* 966 to [LIVTQTMK + 2O]. These marker peptides were also successfully applied for monitoring in the antioxidant study (**II**). Furthermore, the Lys oxidation products to AAS in β-Lg, the ions *m/z* 916 [IDALNENK-AAS] and [GLDIQK-AAS] were found as new possible markers in this thesis study.

Similarly, the promising indicators of α-La oxidation, and thus the marker peptides proposed in this study, were the ion *m/z* 1098 with two different chromatographic compounds (peaks): the peptides [LDQW^{+16}LC^{+48}EK] and [LDQWLC^{+48}EK + O (Asp or Leu)]. For example here the primary oxidation product [LDQWLC^{+48}EK] was considered not stable enough from further reactions for monitoring purposes.

Table 9. The most promising oxidation marker peptides proposed in this research, including their confirmed (in boldface) and tentative identifications.

Identification	$[M + H]^{1+}$	Oxidized residue(s) considered	Stage of oxidation	Mass shift from the intact peptide
α-Lactalbumin				
[LDQW^{+16}LC^{+48}EK]	*m/z* **1098**	**Cys + Trp**	secondary product	+64 u (*m/z* 1034)
[LDQWLC^{+48}EK + O]	*m/z* **1098**	Cys + Asp or Leu	secondary product	+64 u (*m/z* 1034)
[VGINYWLAHK-AAS]	*m/z* **1200**	**Lys**	primary product	-1 u (*m/z* 1201)
[VGINYW^{+16}LAHK]	*m/z* **1217**	**Trp**	primary product	+16 u (*m/z* 1201)
[VGINYW^{+32}LAHK]	*m/z* **1233**	**Trp ×2**	secondary product	+32 u (*m/z* 1201)
[VGINYWLAHK/*N*-fku]	*m/z* **1233**	**Trp**	primary product	+32 u (*m/z* 1201)
[W^{+16}LAHKALC + O]	*m/z* **974**	Trp ×2 or Trp + His	primary product	+16 u (*m/z* 958)
β-Lactoglobulin				
[ALPM^{+16}HIR]	*m/z* **854**	**Met**	primary product	+16 u (*m/z* 838)
[ALPMHIR + O]	*m/z* **854**	His or Pro	primary product	+16 u (*m/z* 838)
[ALPM^{+32}HIR]	*m/z* **870**	Met ×2	secondary product	+32 u (*m/z* 838)
[IDALNENK-AAS]	*m/z* **916**	**Lys**	primary product	-1 u (*m/z* 917)
[GLDIQK-AAS]	*m/z* **672**	**Lys**	primary product	-1 u (*m/z* 673)
[LIVTQTM^{+16}K]	*m/z* **950**	**Met**	primary product	+16 u (*m/z* 934)
[LIVTQTM^{+32}K]	*m/z* **966**	Met ×2	secondary product	+32 u (*m/z* 934)

6 DISCUSSION

6.1 Remarks on MS method development, evaluation of ion suppression and matrix effect

In order to study site-specific oxidation of the whey proteins, new analytical tools were developed and exerted. The adaptation of enzymatic hydrolysis (digestion), fractionation (Prep-HPLC) and oxidation experimentation are explained in brief under their respective titles in Materials and methods (section 4). However, some characteristics of the main analytical tool, the LC–QIT-MS instrument, in reflection to the results obtained are discussed in this chapter.

Matrix effect is always a valid concern in all MS based research, but especially with the ESI technique, as the offered high sensitivity of the instrument depends on its skillful use and understanding of the whole injected sample. Therefore, evaluation of the matrix effect was carefully considered by obtaining baseline chromatograms from the methods developed for both the Prep-HPLC–MS and LC–QIT-MS instruments. None of the sample solutions used were considered very complex, but all solutions used in the studies were still injected and analyzed without the sample components (as 'reagent blanks'). Because no significant solvent background peaks or other disturbance were seen co-eluting with the compounds of interest, the matrix effect was not considered an issue for the created methods. Baseline of all the chromatograms was as level as to be expected for a gradient elution program, with no signs of ion suppression observed when compared to the sample runs. In addition, it was decided not to execute baseline subtraction, since most of the data processing was done from the EIC's.

In order to maintain good analytical practices, the evaluation of possible sample buildup to the analytical systems was continued throughout the analyses. Pure solvent runs ('wash blanks') were injected in every sample batch, usually in the beginning but in some cases also in the end of the set. This also allowed the observations of any possible changes to the gradient baseline, but no substantial inconsistency was noted.

The use of a reference peptide TPEVDDEALEK was considered very useful. Although the method did not aim for quantification, the in-house reference gave invaluable information about the day-to-day variance of the instrumentation and MS-signals in particular. This seems not to be very common in MS-based protein and peptide research, although a similar approach was reported by Le et al. (2013).

The whole experimentation was designed with LC–MS compatibility in mind, including minimizing the use of non-volatile reagents that could lead to contamination of the ion source and to ion suppression with the compounds of interest. In practice, minor contamination of the ion source was observed due to the oxidation sample components, especially the $FeCl_3$-reagent and PIPES-buffer, thus careful cleaning was systematic.

Identification by QIT-MS^n fragmentation

With the QIT-MS-instrument used, the intensities of the fragment ions (favoring of smaller or bigger fragment ions) were greatly influenced by the fragmentation parameters used. In addition, fragmentation often led to losses of side chains from the peptide, and not only cleavage of the peptide bonds, which may have been more ideal for the sequence analyses. Similar behavior has also been previously reported by Qin and Chait (1997). The tendency for complete or partial side chain cleavage was in some cases enhanced by the progression of oxidation status, denoting that addition of oxygen to the side chain increased the likelihood of its fragmentation, and thus the intact peptide and the oxidized peptide did not always produce identical fragment ion intensity profiles for the unaffected amino acids either. Because of this, identification of some of the oxidation products was found challenging. It was relatively easy to observe that modifications have occurred, but the exact placement of the oxidation site could not always be confirmed.

Direct infusion of the samples to the ion source, and thus fragmentation analysis without the HPLC separation, was applied to some of the samples, but it was found useful mainly for the intact peptides. For example the peptide ALPMHIR produced two oxidation products with the same ion *m/z* 854 and the peptide VGINYWLAHK three oxidation products with the same ion *m/z* 1217. Good chromatographic separation of these compounds, instead of direct infusion investigation as one single mother ion, was essential in order to assign the correct fragments to the right oxidation product.

On the other hand the ESI-source of the QIT-MS instrument was found very gentle on the peptide ions with the optimized parameters used, and thus the observed main ions of each compound peak matched the expected molecular ions, and not so much of their smaller fragments. In-source fragmentation was not considered an issue, although some lower intensity fragment ions were in fact detected in the full scan mass spectra for peptides with very high overall concentration. In-source fragmentation can occur inside the instrument, at the stage where the ions are directed from the normal pressure to high vacuum. More precisely, this can happen when the ions exit the capillary and are about to be directed through the skimmers leading further towards the quadrupole ion trap. This region is typically controlled by pre-vacuum pumps, but perhaps not in vacuum high enough for an abundance of ions.

Because the sample flow was directed in-line from the LC, these observed fragments must have originally eluted as a single (full) compound, otherwise the 'peptide fragments' would have been separated by the gradient chromatography. All in all, the observed in-source fragmentation was not considered to cause significant error to the study, especially as identification was always done based on LC–MS^n where the ion trap is operated to produce fragment ions only from a chosen mother ion, and hence the connection is always certain.

As for the newly identified peptide W^{+16}LAHKALC, the occurrence of unoxidized peptide WLAHKALC was extensively searched, but could not be located. If the peptide had been prone to in-source oxidation, two chromatographic peaks with 'W^{+16}LAHKALC' identification would have been seen. However, this was not the case, and only one compound eluted in this one chromatographic peak. It is also noteworthy to mention that with all the other peptides

experimented in the pre- or full oxidation, the unoxidized separate compound peak was always seen. This supports the fact that for some reason the peptide W^{+16}LAHKALC was already oxidized by its Trp side chain in the beginning of these studies.

Protonation and sodium adduct formation

Ionization by protonation of the compounds studied was considered successful. The QIT-MS instrument showed better intensities and signal-to-noise -ratios even in full scan mass spectra than the single-quadrupole MS detector of the preparative HPLC-instrument. Therefore all identifications and assessment of sample purity were done with the LC–QIT-MS, with sensitivity enhanced by analysis from EIC's whenever possible. The occurrence of multiply charged ions was minimal, as was desired by the optimization of the method so that the peptides did not need to be analyzed as a sum of several ion chromatograms. Only the diTyr-dipeptide from VLVLDTDYK showed preference to a doubly charged molecular ion, and was thus monitored as $[2M + 2H]^{2+}$. Surprisingly, it's singly charged molecular ion could not be detected at all even though it did fit in the *m/z*-range used. In contrast, Le et al. (2013) found many of their whey protein-derived peptides to be multiply charged, including for example LDQWLCEK and VLVLDTDYKK, which were both monitored via their doubly charged molecular ions prior to MRM-analysis. They also researched other peptides with familiar fragments, such as ILDKVGINYWLAHK, which had high tendency for a triply or quadruply charged ion.

Trace amounts of sodium could, however, not be avoided in the samples from the lab ware, chemicals and instruments used during the analyses. Therefore an eye was kept on sodium adduct formation for any of the peptide ions studied with the MS. For all peptides except one the relative intensities of the $[M + Na]^+$ ions were small if not trivial, so only the protonated molecular ions could be chosen for monitoring. The only exception was peptide W^{+16}LAHKALC, where the observed strong sodium adduct formation was considered to arise from the helical structure of the peptide. Indeed, a strong helix is located between the amino acids 105-110 in the α-La sequence, while the peptide W^{+16}LAHKALC is positioned 104-111. The same firm helix was thought to possibly enhance the hydrolysis of this peptide as a unit, since it was not an expected hydrolysis product of trypsin. It was not possible to pinpoint the exact position of the sodium, as it is generally not possibly to designate the actual site of protonation in mass spectrometry.

One important point is also that the utilized methods did not aim to quantify the peptides nor their oxidation products, but rather to give a comparison of the phenomena. For this reason the amount of the compounds are presented by their peak areas. If quantitative work is desired in the future, it may be rational to take into account the sum of different forms of ions, i.e. the sum of $[M + H]^{1+}$, $[M + Na]^{1+}$, $[M + 2H]^{2+}$ etc. for each compound, as was done for the peptide W^{+16}LAHKALC. It is not certain that the ratio of all these forms (and other possible adduct ions not mentioned) stays the same between the intact peptide and its own oxidation products.

In these studies the possible inconsistency may explain some of the variance observed between the replicate samples, but was not considered a major problem due to the obvious favoring of the single protonated form $[M + H]^{1+}$, except for the aforementioned W^{+16}LAHKALC.

6.2 Amino acid side chain oxidation

MCO of the amino acid side chain residues was investigated from the tryptic peptides of two whey proteins, α-La and β-Lg. For this purpose, a newly created analytical protocol was employed in which the chosen peptides were oxidized under Fenton chemistry conditions and finally analyzed by LC–QIT-MS^n. Generally, the amino acids of the studied peptides did not show significant oxidation during the sample preparation processes. The only exceptions were Trp in α-La peptide WLAHKALC, where intact peptide was not detected at all, and Met in β-Lg peptide ALPMHIR, where 98% of the intact peptide was lost already on oxidation day 0, compared to the identified oxidation products. Also Met in β-Lg peptide LIVTQTMK showed high tendency for oxidation; the intact peptide detected on day 0 accounted for approximately 10% compared to the oxidation products in study **I** and 1.5% in study **II**. Based on these observations, Met7 and Met145 in β-Lg and Trp104 in α-La may be exceptionally prone to oxidize during processing, compared to all the other amino acids in the peptides studied.

On the other hand, several amino acids in the studied peptides were observed to undergo significant oxidation within time of incubation at +37 °C, of which Cys, Lys, Met, Trp and Tyr were the main targets of MCO. Additionally His and possibly Pro oxidation were suggested. This is well in accordance with the current knowledge (Stadtman 1990; Stadtman and Levine 2003), although it was perhaps found unexpected that Lys oxidation was overall not more dominant, considering it has been suggested as a marker of protein oxidation (Estévez et al. 2009).

The semialdehydes AAS and GGS

The formation of α-aminoadipic semialdehyde from the Lys residues has been used as a target marker of protein oxidation in many foodstuffs, especially in meat products (Akagawa et al. 2006; Armenteros et al. 2009; Timm-Heinrich et al. 2013), but also in bovine α-La (Estévez et al. 2009). However, some studies have presented evidence that Lys oxidation may be remarkably dependent on the oxidative conditions, and MCO may favor other amino acid side chains than Lys. For example in the work of Temple et al. (2006) MCO was seen to affect only two of the 59 available Lys residues in human serum albumin. The same phenomena may well explain why only three of the currently studied peptides showed noteworthy Lys oxidation to AAS, even though Lys was the terminal amino acid in eight of them. Altogether, there are 12 Lys residues in α-La and 15 Lys in β-Lg, both covering nearly 10% of the total sequences. Of these 2 and 6 (from α-La and β-Lg respectively) were covered in the studies and 50%, or more precisely 1 from α-La and 3 from β-Lg, showed some signs of oxidation, even while located as the C-terminus of the studied peptides.

Among the findings of Lys oxidation, only AAS formation from the α-La peptide VGINYWLAHK is proposed as a substantial marker site, even though the sum of detected oxidation products from Trp exceeded the AAS development. This Lys108 is situated at the surface of the α-La tertiary structure, thus offering a potent oxidation site even for undenatured proteins. In addition, the previously unpublished results from β-Lg peptides GLDIQK and IDALNENK support the understanding that Lys oxidation does to some extent occur in whey proteins, but may be less significant compared to other amino acid side chain oxidation reactions. For example in the α-La peptide LDQWLCEK, the existence of Trp and Cys lead to oxidation reactions entirely dominating over those of the terminal amino acid Lys122. Similarly, Tyr oxidation was the main target site in peptide VLVLDTDYK, even though its Lys100 was observed by Fogliano et al. (1998) to be the second reactive Lys residue in β-Lg towards lactosylation, thus fairly solvent accessible. Therefore, the AAS as an only indicator may not be a good marker product for whey protein oxidation. The information gathered in this study indicates that Lys oxidation is often secondary to several other amino acid side chain modifications and even if AAS is detected, some other residues may already be very heavily oxidized.

Two other amino acids capable of oxidizing into carbonyl containing semialdehydes are Arg and Pro. These two share the same end product, GGS, but through different net mass changes; +16 u from Pro and -43 u from Arg (see Table 4.). Both amino acids were part of the same β-Lg peptide sequence, the ALPMHIR, but neither were observed as major target sites. In fact, +16 u was the only observed modification, and thus Arg oxidation was ruled out. In Pro the heteroring contains an amino group which in this peptide is also involved in the peptide bond towards Leu. Considering that the carboxyl-end of Pro is connected to Met, which was highly oxidized, Pro oxidation to GGS could be expected due to close location. Competing with this theory was His, situated on the other side of Met and also with amino-containing hetero-ring available for ROS attack. Unfortunately the exact site of oxidation could not be determined due to pronounced fragmentation of the side chains instead of the peptide backbone, but the observed side chain fragmentation of Δ 28 u could have originated from either, as mentioned earlier in section 5.1. The evidence from Dyer et al. (2016) who identified His146 to oxidize along with Met145, can perhaps be used in favor of His instead of Pro also in this study.

In α-La and β-Lg the total numbers of Arg residues are 1 and 3, respectively, and Pro residues 2 and 8, respectively. None of the remaining Arg were covered in this study, but Pro was part of the sequences in IPAVFK and TPEVDDEALEK, of which the latter was used as a reference peptide due to excellent stability in the pre-testing. Therefore, it can be stated that based on the amino acids capable of forming the semialdehydes in these studied peptides, neither AAS or GGS seem to be characteristic oxidation products in whey peptides.

Methionine, the main target site

In terms of oxidation susceptibility, Met was found to be the most prone amino acid to oxidize in both of the peptides it was present. These were located as Met7 and Met145, both in β-Lg. Altogether 4 Met residues are situated in the whole β-Lg protein. Only one Met residue exists in α-La, Met90 next to a cross-linked Cys91, and this part of the sequence was not included in the studied α-La peptides. However, Met90 has previously been shown to be a considerable site of oxidation by two different approaches, accounting for +16 u mass additions by both methods (Dalsgaard et al. 2013 and 2014).

In the proposed marker peptide LIVTQTMK, oxidation of Met7 to sulfoxide and all the way to Met sulfone dominated and no significant Lys oxidation was observed within the 2-week experimentation. Met145 in peptide ALPMHIR was almost completely in sulfoxide form already on day 0, and the trend was decreasing strongly towards day 4, at approximately the same rate as the dioxidized form of the peptide was forming. Same strong sulfoxide formation of Met7 was also identified by Meltretter et al. (2007) in their peptide LIVTQTMKGL incubated with lactose and a thermal treatment. They also reported a further +16 u mass addition to some of their Met containing peptides, but modification to Met sulfone was not confirmed. Later Met sulfoxide formation profiling studies have revealed all of the named sites; Met7 and Met145 in full β-Lg and Met90 in full α-La, to be prone to H_2O_2 oxidation in the absence of added metal ions (Wüst and Pischetsrieder 2016). In addition, Dyer et al. (2017) have recently observed dioxidation in some Met residues of β-Lg, but did not identify if Met7 or Met145 were one of them, although did report significant sulfoxide (monoxidation) formation from both.

The many stages of cysteine and tryptophan oxidation

Although prevailing in both whey proteins, the Cys and Trp residues covered by these studies were only present in the α-La-derived peptides, corresponding to Trp104, Trp118, Cys120, and Cys111. All of the 8 total Cys residues are bound by intra-molecular disulfide bonds in intact α-La, but one of the 5 Cys residues of β-Lg has a free thiol group (either Cys119 or Cys121, not covered in the studies). The studied two Trp residues represented 50% of total Trp in α-La, while β-Lg also would have had two.

Cys120 in peptide LDQWLCEK showed rapid trioxidation (+48 u) to Cys sulfonic acid (cysteic acid) during the first 24 h of oxidation. Also Trp118 in the same peptide would have been capable of trioxidizing, but in this case the two targets were distinguished from each other by the remaining fluorescence properties of the peptide. Finley et al. (1998) showed that loss of fluorescence is a typical consequence of Trp oxidation, and this has since been successfully applied to monitoring purposes, such as monitoring of whey protein emulsion stability (Viljanen et al. 2005). Thus, in peptide LDQWLCEK the trioxidation was occurred in Cys. This is further in line with the studies of Claiborne et al. (1999) who stated that Cys sulfonic acid is a relatively stable end product. Indeed, from day 1 onwards the profuse oxidation of

Cys could be seen by the effect to the nearby amino acid residues, causing two different secondary oxidation products with additional +16 u. At least one of these showed the expected loss of Trp and was thus assigned to 5-OH-Trp (or one of its isomers, 2-/4-/6-/7-OH-Trp).

Cys111 was the C-terminal amino acid in peptide W^{+16}LAHKALC, and overall this peptide turned out to have several highly prone target sites producing multiple combinations of oxidation modifications that did not produce any major ions to be monitored within time. Cys111 was considered to have major share of the modifications, but the products could not be confirmed. The final conclusion was that the Cys mono-, di- and trioxidation products were considered uncharacteristic to be used in marker peptides, even though Cys111 was most likely a typical oxidation site to explain the deterioration of the intact peptide form. Cys111 and Cys120 were nominated as oxidation sites also in the work of Meltretter et al. (2007)

Trp104 and Trp118 were both eager targets of MCO. Trp104 was the N-terminal amino acid in W^{+16}LAHKALC, where monoxidation was present already since digestion and fractionation of the peptide. The same Trp104 was located in the middle of peptide VGINYWLAHK, where no artefactual oxidation was present, but W^{+16} was formed during the incubation. In fact, the Trp residue of this peptide showed several oxidation modifications, some seen with the same *m/z*-ratio ion but separated by chromatography. The identifications named in study **III** were tentative, as the method used could not confirm the exact position of added oxygen. For example, 5-OH-Trp is perhaps the best known isomer in food-related literature (van Wickern et al. 1997; Salminen 2009), but the thus named compound (item C in Figure 13.) could also arise from the other isomers: 4-, 6- or 7-OH-Trp. Likewise, Oia (item A, Figure 13) was designated based on strong loss of fluorescence properties due to the carbonyl formation in position 2 in the pyrrole moiety, and the close-eluting matching secondary oxidation products following from Oia; the diOia and *N*-fku.

The many oxidation modifications arising from Trp, although not all named with full certainty, can be of health concern in food products. Both kynurenine and *N*-fku have been connected to urinary bladder carcinogenesis in mice, as suspected promoters of onset, and other carcinogens arising from Trp have also been named (Matsukura et al. 1981; Krogul and Fennema 1987; Stadtman and Berlett 1997). Another fact of concern is that the oxidation products of Trp have been shown to be more susceptible to further modifications than Trp itself, and thus it is of importance to minimize the beginning of any such reactions (Itakura et al. 1994; Simat and Steinhart 1998).

During the studies, one of the oxidation products from Trp104 in α-La peptide VGINYWLAHK was indeed assigned to *N*-fku, with the matching mass addition of +32 u as demonstrated by Domingues et al. (2003). This compound was pronounced in the samples from day 4 onwards, with no significant deterioration towards the end at day 14. Similar *N*-fku formation was not observed from Trp118, even though its monoxidation did occur. However, it is possible that *N*-fku could develop if the time of oxidation was longer or oxidative conditions harder, but under the current conditions used, oxidation of Cys120 dominated over Trp118 in peptide LDQWLCEK. This is in line with the studies of Dalsgaard et al. (2007) who found that highest content of *N*-fku had formed in α-La, of all the studied dairy globular proteins exposed to photo-oxidation.

Furthermore, the oxidation of both Cys and Trp residues may be of high importance in actual dairy products, as indicated by Elias et al. (2005) who found both of these amino acid residues to be depleted even before the onset of lipid oxidation in their β-Lg protein-emulsion studies. This study confirms both of the aforementioned amino acid residues as very typical target sites of MCO, and the formation of their many modifications seems to be accelerated along with a more accessible position in the sequence.

Tyrosine, the single marker target

Oxidation of Tyr99 in β-Lg, and its formation to diTyr, was the only confirmed Tyr modification in the study. Tyr103 present in one of the α-La peptides did not seem to be prone to oxidize. The same finding was reported by Dalsgaard et al. (2014) who postulated that Tyr103 seems to be exceptionally stable against oxidation, which may be due to the protective location towards the inner core of the protein, or possibly by the high vulnerability of the neighboring two tryptophans (by sequence and by tertiary folding).

However, of the four Tyr residues in β-Lg, Tyr99 and Tyr20 are solvent accessible, while Tyr42 and Tyr102 are rather buried into the core, as first found by Townend et al. (1969) and later identified by Brownlow et al. (1997). Monitoring of the observed diTyr formation from Tyr99 could thus serve as an excellent indicator of oxidation in this protein region. Since no other prominent oxidation products were found from peptide VLVLDTDYK, Tyr was the confirmed main target site, also over Lys. Information of diTyr formation was evaluated from specific wavelengths of both FL and UV, in addition to the generally used 214 nm (UV). According to Giulivi et al. (2003), the λ_{max} for Tyr are 234 and 274 nm, and the λ_{max} for diTyr are 236 and 284 nm under acidic conditions (pH 3.1). In the studies of peptide VLVLDTDYK data was evaluated from 235 and 274 nm, which did confirm the presence of Tyr, but did not give much additional information. Instead, the use of fluorescence detection is strongly advised over the aforementioned UV monitoring, based on these findings. The diTyr-dipeptide formed from VLVLDTDYK showed highly pronounced fluorescent properties, which would give an indication of oxidation even at low level. However, this also emphasizes the need of careful calibration of the fluorescent standard, as even small amounts formed could be misinterpreted as ‘major findings’ because of the high signal. On the downside, the choice of fluorescence wavelengths is quite strongly pH-dependent, as demonstrated by Giulivi and Davies (1994); excitation should be done either 315 nm (alkaline) or 284 nm (acidic) to produce the intense 420 nm emission, but other components of the food sample may also effect the result.

6.3 Protein oxidation marker peptides

As presented in the previous chapter, several amino acid side chains were detected to suffer oxidative alterations. One of the main interests in this study was to investigate if promising marker peptides could be identified as indicators of oxidation. The main criteria for determining if a peptide could serve as a potential marker were (i) the susceptibility of certain known amino acid(s) to oxidize and (ii) to preferably form a peptide product that is stable enough for monitoring purposes. Therefore, it is important to distinguish that the hereby proposed marker peptides do not always represent the amino acid residues observed to be *the* most prone for oxidative modifications in their respective peptides.

For example in peptide LDQWLCEK it was discovered that Cys was highly prone to oxidize rapidly all the way to cysteic acid (+48 u), which induced rapid further oxidation of e.g. Trp. Therefore the primarily formed peptide [LDQWLC^{+48}EK] itself was not considered a promising stable marker peptide, even though this Cys120 was proposed as one of the main target sites of MCO in α-La. Instead, it is suggested that a mass shift of +64 u would be better for monitoring as an indicator of oxidation from the intact peptide LDQWLCEK (mother ion *m/z* 1034). The peptide LDQWLCEK is especially interesting because it contains both of the negatively charged amino acids Asp and Glu. They are known to be excellent binders of transition metals (Bertini and Turano 2007), which means that this region does not have to be available on the surface of the protein to be prone to oxidize. This may well explains the detected high reactivity of both Cys and Trp.

Similar high oxidative reactivity of Cys111, as well as Trp104, was observed for the α-La peptide [W^{+16}LAHKALC]. This peptide could not even be fractionated without observing Trp monoxidation, and the Cys modifications followed soon after the Fenton reagents were introduced in the oxidation study. While the many combinations of oxidized amino acid residues were a challenging task to identify, one peptide product was detected forming throughout the 2-week experimentation. This potential marker peptide was monitored as an addition of +16 u to the peptide that already included the monoxidized Trp. Unfortunately the product, [W^{+16}LAHKALC + O], could not be reliably identified, but the oxygen addition was postulated to arise from His oxidation to 2-oxo-His. The study of Schöneich (2000) proposes that His oxidation to 2-oxo-His is very site-selective, thus dependent on the surrounding sequence, and the Fenton chemistry conditions used in this thesis study match the preferred environment for this modification. The formation of 2-oxo-His from the His residue would also possess an excellent marker modification because it is not very prone to oxidize further, thus if formed also easily monitored within time.

The α-La peptide VGINYWLAHK was partly overlapping in sequence to W^{+16}LAHKALC. Several potential marker peptides were proposed for the first-mentioned peptide, where several modifications of Trp could be chromatographically separated from each other. The supporting information gathered from the separate oxidations of these two peptides are first of all in line with the current understanding that the sequence environment has an influence on the modifications formed of the individual residues. In this study the influence was observed as rate of modifications formed, rather than type or susceptibility for oxidative changes. In peptide

W^{+16}LAHKALC, the expected main targets sites Trp and Cys were located on both ends of the peptide, and thus freely accessible to the oxidizing conditions and resulted in multiple combinations of their respective modifications. In peptide VGINYWLAHK Trp oxidation to W^{+16} and beyond could be witnessed more systematically, which could result from the absence of Cys that was possibly accelerating the ROS formation reactions, or the different location of Trp from the N-terminus to the middle amino acid in the peptide. On the other hand, neither of the peptides are very long by sequence, both around 1 kDa, so steric hindrances should not be a significant issue.

Also interestingly Lys oxidation to AAS from VGINYWLAHK turned out to be an excellent marker for this peptide, and not least because of its excellent chromatographic separation, which would benefit any monitoring purposes. In W^{+16}LAHKALC the AAS formation was a minority again compared to Trp and Cys, but it may be worth to search this marker possibility in a longer sequence, for example 'VGINYWLAHKALC'. This sequence region may well be of high importance in full proteins, because it has already been shown to be one of the main sites to increase in accessibility due to photo-oxidation induced unfolding of α-La (Dalsgaard et al. 2008).

The proposed most potential markers of oxidation from β-Lg include sequence regions ALPMHIR and LIVTQTMK. In addition, promising markers were found to be Lys14 and Lys91 oxidation to AAS in peptides GLDIQK (the AAS-peptide to monitor with *m/z* 672) and IDALNENK (the AAS-peptide seen with *m/z* 916), respectively.

For the sequence region 142-148 the proposed markers were ALPM^{+16}HIR and [ALPMHIR + O] (both monitored with the ion *m/z* 854 instead of the intact *m/z* 838), as well as the doubly oxidized ALPM^{+32}HIR (ion *m/z* 870). From sequence region 1-8 the proposed two markers were both monoxidation and dioxidation of Met, namely the peptides LIVTQTM^{+16}K (ion to monitor *m/z* 950 instead of the intact *m/z* 934) and LIVTQTM^{+32}K (seen with ion *m/z* 966). Especially all the three oxidation products observed from peptide ALPMHIR were more or less increasing from day 4 onwards, emphasizing the importance of this peptide region in the full protein sequence.

These results are supported by the findings of Meltretter et al. (2007), which confirm that the same target sites seem to prevail even in their longer peptides. Among those findings were Met oxidation to sulfoxide in the AspN hydrolyzed β-Lg peptides LIVTQT**M**KGL and DKALKALP**M**HIRLSFNPTQLEEQCHI, but the experimentation included only added H_2O_2 and no metal ions. In addition, the peptide ALPM^{+16}HIR and another nearly identical peptide to the one in this dissertation (study **I**), peptide ALIVTQTM^{+16}K, have only recently been suggested as promising markers of protein oxidation in milk whey proteins by Dyer et al. (2017). This could perhaps be considered as a proof of concept, and that there is up-to-date research interest as well as need for such markers to be used in future applications. Several of the peptides proposed in this dissertation have already been used in further interaction studies, for example with lipids and phenolics carried out by Gürbüz and Heinonen (2015), and Wang and Heinonen (2017).

6.4 Oxidation in reflection to the full whey proteins

The effect of photo-oxidation on milk and dairy proteins has been rather widely studied, and is several times reflected throughout this thesis study. However, direct comparison is problematic, since the main targets of photo-oxidation (both UV and VIS) are known to be the intrinsic chromophoric amino acids, mainly Trp and Tyr (Pattison et al. 2012). Photo-oxidation reactions in foods also always include other ingredients with bound chromophores, for example flavins and porphyrins, and their reactions in either accelerating or quenching the oxidation reactions may be equally important. For example His, Phe, Cys and Met are known to oxidize mainly by the ROS generated *via* other photo-oxidation reactions, and less significantly by direct UV damage (Pattison et al. 2012). Naturally, photo-oxidation is an important mechanism to understand in milk and many other dairy products, and has been studied with increasing interest (Dalsgaard et al. 2007; Scheidegger et al. 2010). However, the MCO reactions may be of higher importance in further processed dairy food products, as well as whey protein concentrates, although less researched.

This thesis study brings new information to the table in whether completely different oxidation sites or fairly dissimilar preferences in β-Lg and α-La exist due to MCO. In reality, metal ions are readily available in the foodstuffs and it may be most appropriate to consider a combination process, such as photo-induced metal-catalyzed oxidation, where understanding of both mechanisms are important. The closest combination so far may be that of Scheidegger et al. (2016), who studied the influence of Fenton chemistry components (H_2O_2, Cu^{2+} and Fe^{2+}) and photo-oxidation to the main dairy proteins.

For any of the previously named amino acid residues and peptide sequence regions to participate in oxidation reactions, some accessibility is needed. Generally, those residues located on the surface of the protein are more likely to participate in oxidation reactions. However, controversial evidence exists whether these modifications are actually significant to the actual activity of the protein, or if some kind of sacrificial protection exists (Törnvall 2010). Therefore the detection of oxidation modifications in the "inner" amino acid residues may be of higher importance, as those would indicate the opening of the tertiary folding and occurrence of reactions perhaps more substantial to the actual value of the protein. This could be seen for example by loss of technological function, such as solubility, emulsifying or water-binding properties, or by deterioration of the nutritional quality. For example Met oxidation to Met sulfoxide significantly increases the hydrophilicity of its original location, and most likely leads to loosening of that region.

However, in MCO the most susceptible amino acid residues do not have to be located on the surface of the protein (Schöneich 2000). The reactions initiated by for example processing can first accelerate within a small closed region next to the bound transition metals, and where the increasing level of oxidation then promotes loosening of that part of the protein structure. Typically the Cu- and Fe-ions are bound within 4-6 amino acid side chains of a protein, such as Cys, Met, His, Glu, Asp or Tyr (Bertini and Turano 2007). Even though the negatively

charged Glu and Asp are not very prone to oxidize themselves, their presence may well promote oxidation in the susceptible residues nearby. This feature can especially be connected to the α-La derived peptide LDQWLCEK of this study.

In addition to the proposed marker peptides being more or less good representatives of MCO modifications in their sequence region, some of the very same peptides are of research interest due to their bioactive properties. These studies have evolved especially around the β-Lg peptide ALPMHIR (Mullally et al. 1997; Ferreira et al. 2007; Chatterjee et al. 2015) and α-La peptide VGINYWLAHK (Pihlanto-Leppälä et al. 2000; Chatterjee et al. 2015), both showing ACE-inhibitory effect and the latter also antimicrobial and antioxidative properties. Especially the anti-hypertensive possibilities of ALPMHIR, the also named lactokinin, have been studied as an example of the many possibilities of the milk protein-derived peptides as components of functional foods and future pharma- & nutraceuticals (Maes et al. 2004; Pina and Roque 2009). This thesis study can offer important information of these same peptides and their susceptibility to structural alterations, to be taken into account in future product development. An example of a potential benefit can be seen with the work of Chobert et al. (2005) who studied the possibility to enhance the bioactivity of ACE-inhibitory peptides in fermented milk and yoghurts. Their findings include for example that α-La was better hydrolyzed than β-Lg during the fermentation of yoghurts, despite of the different starters used. Fermentation of milk increased the formation of ACE-inhibitory peptides and their activity, compared to activity measured from β-Lg hydrolyzed with trypsin alone. However, they conclude with the observation that some kind of "non-specific inhibition" occurred in the fraction of proteins and peptides where most ACE-activity was expected. This named "great inhibition" of the first-observed ACE-activity could result from oxidation of the amino acid residues in sequence parts where the ACE-inhibitory or antimicrobial activity is also connected to, such as Trp, Tyr, and Phe.

Another aspect of the nutraceutical industry is the ever-growing interest in whey proteins as part of a sports nutrition. Many sports powders and protein isolates are made of milk whey, due to their excellent nutritional value, but not much is known about the effect of processing to the end products. Thus, new analytical tools would be needed for better evaluation of the actual nutritional quality. Indeed, it should not be forgotten that results greatly depend on the fitting of tools used. Semagoto et al. (2014) studied UV-induced photo-oxidation in a milk protein concentrate, and found loss of solubility for several of the proteins due to aggregate formation by non-disulfide covalent cross-link formation. Additionally, it was concluded that the unstructured casein was more susceptible to oxidation than β-Lg, but this was monitored by the carbonyl formation. This thesis study did not compare caseins to the whey proteins, but suggests that the non-carbonyl oxidation products indeed play an important role in whey proteins, for which evidence has been presented. For example the data of Moskovitz and Oien (2010) suggest that Met oxidation may actually be regulating the pathway leading to the formation of protein carbonyls, thus, in Met-containing foods such as milk products this may be of significance.

7 CONCLUSIONS

In this study, metal-catalyzed oxidation (MCO) was investigated in peptides hydrolyzed from the two whey proteins in bovine milk, β-lactoglobulin and α-lactalbumin. For this purpose, a novel analytical approach was created benefiting especially from the available and invaluable mass spectrometric tools. The established protocol consisted of in-liquid digestion with modified trypsin, separation and fractionation of the chosen peptides with preparative-HPLC–MS, followed by individual oxidation and analysis with the designed LC–ESI-QIT-MS methods. This combination of the various tools was proven to be functional by the encouraging results obtained in the study.

Trypsin was found to produce several peptides of practical size for the MS analyses, and all of them could be seen to ionize in a single charged form for feasible monitoring. The peptides chosen for oxidation analysis were selected based on their amino acid composition, with at least one component known to be prone to oxidize by the Fenton chemistry conditions used. Lys was the C-terminal amino acid in most of the studied peptides due to the typical cleavage of trypsin, but interestingly, only 50% of the studied Lys residues were seen to show some degree of oxidation to the expected typical modification, the α-aminoadipic semialdehyde (AAS). In addition to AAS, Arg and Pro oxidation to a similar γ-glutamic semialdehyde (GGS), was found marginal. Both AAS and GGS have previously been used as markers of oxidation even for the milk proteins. According to this study, monitoring of AAS formation may be a good site-specific marker, such as for Lys108 in α-La peptide VGINYWLAHK. However, the information gathered in this study indicates that even if Lys oxidation is detected, several other residues have already oxidized more significantly. Therefore, the estimation of overall oxidation status using the abovementioned semialdehydes cannot be considered characteristic, and most likely leads to a significant understatement for these studied whey proteins.

Among the observed most susceptible target sites of MCO in the peptides studied were the amino acid side chains of Met, Cys, and Trp. Several of the proposed marker peptides had at least one of these residues oxidatively modified. In at least one of the proposed potential marker peptides, the α-La peptide [LDQW^{+16}LC^{+48}EK], Trp oxidation was observed secondary to Cys trioxidation all the way to cysteic acid.

Met oxidation to sulfoxide was the confirmed MCO target site in β-Lg peptides ALPM^{+16}HIR and LIVTQTM^{+16}K. Both of these Met residues have previously been reported to be prone to photo-oxidation reactions, and the current findings confirm that these proposed marker peptides have high potential in future applications. MCO in general is a more site-specific process than photo-oxidation, due to the involvement of transition metals often bound to certain amino acid regions. Therefore, MCO reactions and the oxidation modifications reported here are more likely to occur even in the inner hydrophobic regions of the whey proteins, thus leading to partial unfolding of the tertiary structure, and offering the exposed sequence regions available to further modifications. This can be of significance to the technological properties of the whey proteins, and by minimum, result in loss of nutritive value in the affected amino acid residues.

This dissertation study has offered several confirmed target sites of MCO in the two main whey proteins, α-La and β-Lg, which can be benefited in future targeted analysis with further-developed MS based tools. In addition, the tryptic target peptides observed to produce relatively stable, yet substantial oxidation modifications, were suggested for monitoring purposes. Some of the peptide markers proposed in this study have since then already been used for successful monitoring against a phenolic antioxidant, as well as in lipid interaction studies. Thus, both the findings as well as the analytical tools developed in this study offer a sound basis for further investigations.

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